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The production of glutamic acid by fermentation

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THE PRODUCTION OF GLUTAMIC ACID BY FERMENTATION

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ABSTRACT

The method of producing glutamic acid by fermentation was studied in shake flasks and in a five liter fermentor. Strains of the micro-organism, Micrococcus glutamicus, were used to produce glutamic acid under different conditions.

The five liter mechanically agitated fermentor used in this investigation was constructed in the machine shop of the Department of Chemical Engineering and Chemistry by the author.

The pH, residual glucose and glutamic acid concentration were determined during the fermentations. Dissolved oxygen in the medium was measured by a specially designed oxygen analyzer and relationship between dissolved oxygen concentration and the incubation time was obtained.

An optimum biotin concentration, 2.5 γ/l, for glutamic acid production in shake flask was obtained. Indications that leucine was formed besides glutamic acid and glutamine in the fermentation broth were observed.

A literature review of the general properties of glutamic acid and industrial methods of glutamic acid production was made. A detailed discussion of the fermentation method of glutamic acid production was also included.

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I. INTRODUCTION

The microbiological methods of producing foods and beverages were unconsciously applied by our ancestors. They made wine from fruit juice and baked bread with yeast. Today we still use these methods in producing many substances. This is because the fermentation method is the easiest way of producing foods, beverages and even more complex compounds, which we are unable to manufacture economically by synthetic methods.

In the last twenty years fermentation has expanded its application to the production of antibiotics and amino acids. These new applications have contributed to the well being of mankind.

One of the most successful achievements in amino-acid production by fermentation is the microbiological production of L-Glutamic acid. Glutamic acid was discovered by Ritthausen in 1866. In 1908 professor Ikeda found that the material enhancing the taste of sea weed (tangle) soup came from a sodium salt of glutamic acid (monosodium glutamate). Since then monosodium glutamate has been widely used in both the food industry and by the general public as a flavor-enhancing agent. The demand of glutamic acid has increased rapidly. Before 1956 glutamic acid was mainly obtained by the hydrolysis of plant proteins, e.g. gluten.

In 1956 Japanese investigators reported the possibility of

producing glutamic acid by microbiological means, and began on an industrial scale the production of this amino acid. Because of their success, this method has been accepted by other nations, and also has stimulated investigators to find other micro-organisms and methods to produce glutamic acid more effectively.

Though the recent interest in the manufacture of glutamic acid has shifted to chemical synthetic methods, the microbiological method of producing glutamic acid accounts for nearly all of the glutamic acid used today. Despite the widespread use of the microbiological method for the production of glutamic acid little has been published until recently on the conditions required for its production.

In this thesis the author studied the conditions required for the accumulation of glutamic acid by Micrococcus glutamicus in a glucose-ammonium medium, and evaluated the relation between the rate of oxygen comsumption and the growth of the micro-organism.

A five liter pilot plant scale glass fermentor and the auxiliary equipment was constructed; and the fermentations were performed in this fermentor and in shake flasks under various conditions. The conditions studied were cell strain, medium composition, antifoam additive concentrations, aeration, agitation and fermentation time.

II. LITERATURE REVIEW

History and Isolation of Glutamic Acid from Natural Sources

In 1866, while engaged in the study of various vegetable proteins, Ritthausen (1866) hydrolyzed wheat gluten with sulfuric acid. At the end of the reaction calcium hydroxide was added in excess to precipitate sulfate, oxalate in excess to precipitate the calcium, lead carbonate in excess to precipitate oxalate, and hydrogen sulfide in excess to precipitate the lead. The final, clear flitrate was concentrated until crystals of tyrosine mixed with another and more water soluble substance separated. On careful treatment with warm water, the latter was separated and was deposited from the chilled, aqueous extract in crystalline form. This turned out to be a new amino acid which, in view of its source, Ritthausen named glutamic acid. In 1873, Hlasiwetz and Habermann (1873) used hydrochloric acid instead of sulfuric acid as an agent for the complete hydrolysis of proteins and obtained a hydrochloric acid salt of L-Glutamic acid. Due to its insolubility in hydrochloric acid solution which far exceeded that of any other amino acid hydrochloride, it was easily isolated from protein hydrolysates.

L-Glutamic acid and its derivatives exist widely in nature. However, only a small quantity of D-glutamic acid is found in the free state, such as, in fresh milk, in human urine, and traces in plant organs.

Glutamine ($H_2NCO \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$), the amide of glutamic acid is common in the botanical field.

Properties of Glutamic acid

Chemical names. (Greenstein and Winitz, 1961)

2-Aminopentanedioec Acid
1-Aminopropane-1:3-dicarboxylic Acid
α-Aminoglutaric Acid

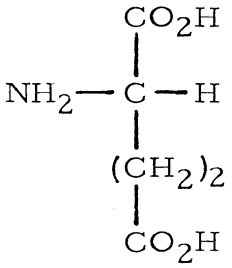
Trivial name. Glutaminic Acid

Empirical formula.

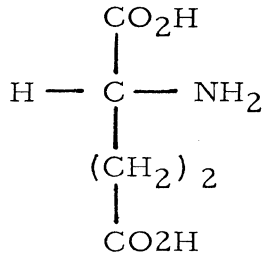
$C_5H_9O_4N$; C 40.82%, H 6.17%, O 43.50%, N 9.52%

Molecular weight. 147.14

Structure and designation of stereoisomers



L-Glutamic acid
l (+)-Glutamic acid
d-Glutamic acid



D-Glutamic acid
d(-)-Glutamic acid
l-Glutamic acid

Density. 1.460

Melting point. l-Glutamic acid 202-203°C
dl-Glutamic acid 199°C

Isoelectric point. pH = 3.22

Physical properties of glutamic acid

Optical rotation. Glutamic acid is an optical active substance.

It appears dextrorotary (d) in acid solution and changes to levorotary after neutralization. Several specific optical rotation values were reported; Ritthausen (1869) $[\alpha]_D = +34.7^{\circ}$ in dilute nitric acid, Schulze and Bosshard (1886) $[\alpha]_D = 31.7^{\circ}$ in hydrochloric acid solution.

Solubility of glutamic acid. The solubility of glutamic acid varies with the pH and temperature of the solution. The solubility decreases to a minimum at its isoelectric point of pH 3.2. At pH value between 2.5 and 3.5 the difference in solubility is small and increases suddenly beyond this range. Hence, the precipitation of glutamic acid should be done at pH 3.2 or at least in the range of 2.5 to 3.5.

The solubility of L-Glutamic acid at pH 3.2 can be denoted by the following data (Seidell 1941).

Table 1. The Solubility of L-Glutamic Acid at pH 3.2

Temperature ^{o}C	Solubility g./l.
0	3.41
25	8.64
50	21.90
75	55.30
100	140.00

Chemical properties of glutamic acid

Biochemical reactions. L-Glutamic acid is readily decomposed

or converted to another compound by the presence of enzymes.

a. Transamination: The NH_2 group of L-Glutamic acid is transferred to a keto acid by the reaction of transaminase.

$$\text{L-Glutamic acid} + \text{oxaloacetic acid} \xrightarrow{\text{Transaminase}} \text{α-Ketoglutaric acid} + \text{L-Aspartic acid}$$

b. Deamination

$$\text{Glutamic acid} + NAD^+ + H_2O \xrightleftharpoons{\text{deaminase}} \text{α-Ketoglutaric acid} + NADH + H^+ + NH_3 \quad *$$

c. Decarboxylation (Gale 1945)

$$\text{L-Glutamic acid} \xrightarrow{\text{decarboxylase}} \gamma\text{-aminobutyric acid} + CO_2$$

d. Glutamine synthesis

$$\text{Glutamic acid} + ATP + NH_3 \xrightarrow{Mg} \text{Glutamine} + ADP + P_i \quad *$$

Hence, in food seasoning, when using monosodium glutamate care should be taken regarding these enzyme and biochemical reactions. It is better to add it at the end of cooking, fermentation or long periods of storing.

*Abreviations used in this thesis:

NAD:	nicotinamide-adenine dinucleotide
NADH:	reduced micotinamide-adenine dinucleotide
ATP:	adenasin triphosphate
ADP:	adenasin diphosphate
Pi:	phosphoric acid

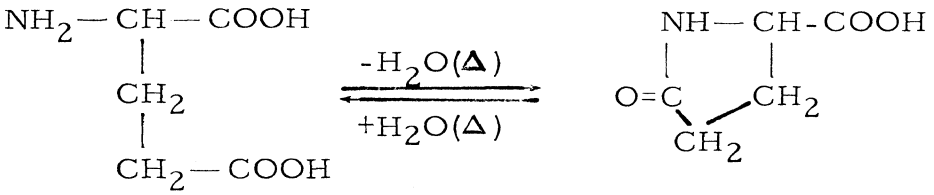
Chemical decomposition. L-Glutamic acid can be oxidized in air at high temperature as the following reaction.

$$HOOC\text{-}CHNH_2\text{-}CH_2\text{-}CH_2\text{-}COOH \xrightarrow{O_2 + H_2O} HOOC\text{-}CH_2\text{-}CH_2\text{-}COOH + CO_2 + NH_4OH$$

However, at low temperature, oxidation does not occur even when it is exposed to air for a long period of time. (Greenstein, 1961)

Racemization. When natural glutamic acid was treated at 150-160°C with an excess of barium hydroxide for several days, the optical rotation disappeared and racemic glutamic acid could be prepared (Greenstein and Winitz, 1961). This preparation was a mixture of equal amounts of L- and D-forms. Racemization is accelerated by strong acid and especially by strong basic solution, and also at high temperature.

Condensation. Glutamic acid can be converted to pyrrolidone carboxylic acid under vigorous conditions.



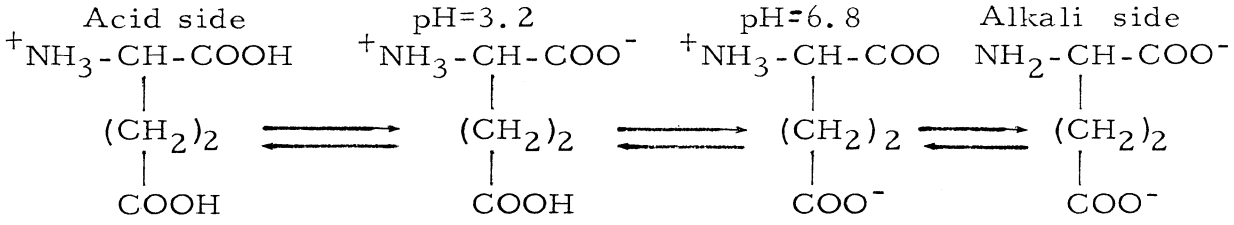
Wilson and Cannan (1937) observed that, in less than 50 hours at 100°C, 98% of the original glutamic acid was converted to pyrrolidone carboxylic acid in an aqueous medium at pH 4 or at pH 10. At 120°C

and pH 3, complete ring closure (100% of original glutamic acid) occurred in less than 3 hours.

Of equal interest is the fact that L-pyrrolidonecarboxylic acid can be reconverted by adding strong HCl to L-Glutamic acid. The reverse reaction, whereby the ring is completely opened, can be effected by 2N HCl or 0.5N NaOH in 1 to 2 hours at 100°C.

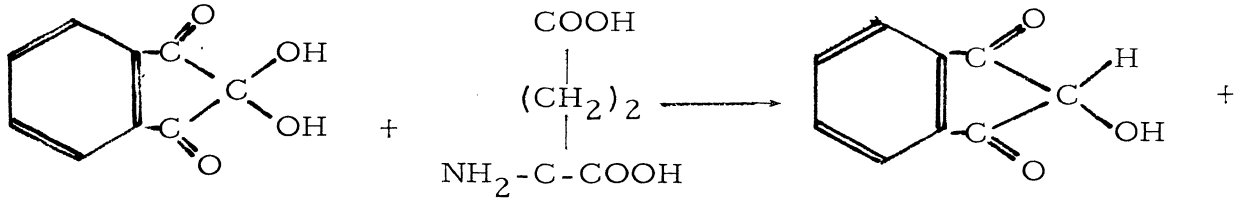
Electrolysis

L-Glutamic acid in an aqueous solution is considered as an electrolyte, such as in the following: (Greenstein 1961)

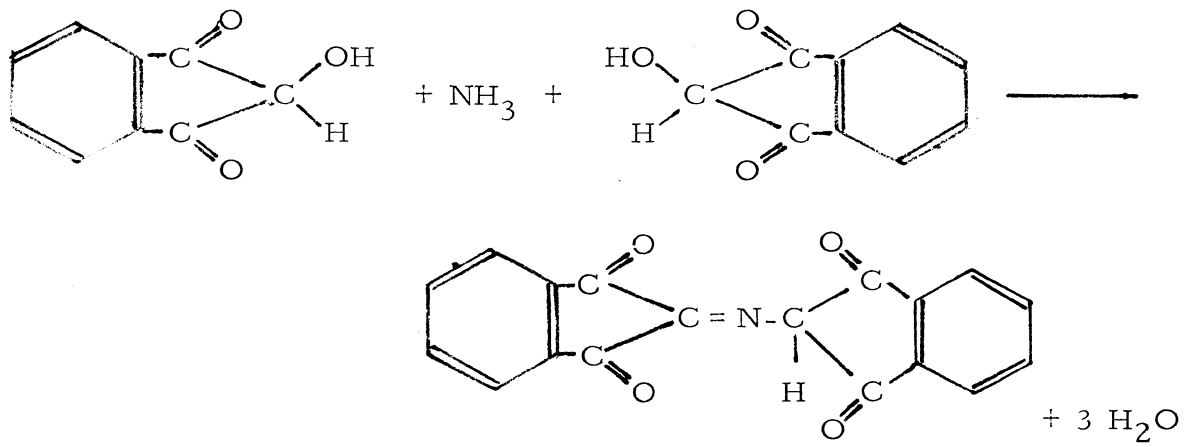


Color reaction

Glutamic acid gives a blue color when it reacts with ninhydrin (triketohydrindend hydrate) (Fruton and Simmonds 1958)



COOH
|
$(CH_2)_2CHO + CO_2 + NH_3$



Glutamic acid will form a precipitation with most basic dyes such as Crystalviolet, Nilbran, and Serfranin.

Glutamic acid Hydrochloride

The complete hydrolysis of protein with hydrochloric acid yields glutamic acid as a hydrochloric salt. This salt has a strong acidity and slight taste. Pure glutamic acid-hydrochloride has 7.63% (wt.) N and 19.33% (wt.) Cl, and the specific optical rotation is shown as below:

Table 2. The Specific Optical Rotation of Glutamic Acid Hydrochloride

Concentration wt. %	$18^{\circ}D$
10.0	+28.63
5.0	+27.45
2.5	+26.85
0.5	+22.87

The crystalline glutamic acid-hydrochloride is colorless and its melting point (decomposition) is 150-160°C. The solubility of

this salt is shown as below: (Ogawa and Fuji 1949).

Table 3. The Solubility of Glutamic acid-hydrochloride in water.

Temperature °C	Solubility g./l
0	298
25	479
50	769
75	1240
100	1990

Industrial Methods of Preparation of Glutamic Acid

Since 1908, monosodium glutamate has been used as a flavor enhancing agent both by the food industry and the general public. The demand for glutamic acid has increased rapidly. The demand has stimulated numerous investigators to find convenient and economical ways of obtaining glutamic acid. However, these are published in various patents or are closely guarded industrial secrets.

During the past fifty years, glutamic acid was obtained mainly by the acid hydrolysis of plant proteins. However since 1956, the microbiological method has begun to supplant the old method (Kinoshita 1963). Recently, it was reported that in Japan a synthetic method of producing this amino acid has been developed and a new plant for its manufacture has been established. (Oil, Paint and Drug Reporter 1962).

The industrial methods for the production of glutamic acid can be classified into 1) hydrolysis of protein compounds. 2) microbiological fermentation. 3) synthetic method.

Hydrolysis of protein compounds

a. Acid hydrolysis of protein rich compounds. The raw materials used in this process are those proteins rich in glutamic acid, such as gluten, soybean cake, or casein. The raw material is hydrolyzed by concentrated hydrochloric acid in corrosion resistant kettle (either glass or rubber lined) for five to six hours at 110°C. When the protein is completely hydrolyzed, the charcoal like humus is removed by filtration. The filtrate is then concentrated in vacuo. The concentrated syrupy liquid is cooled down to -5°C for several hours. During the process of cooling the hydrochloric acid salt of L-Glutamic acid crystallizes. The crystalline glutamic acid is separated from its mother liquid by mechanical means. The glutamic acid-hydrochloride is re-dissolved in hot water. Iron is removed from the salt solution and the solution is then de-colorized with sodium sulfide and activated charcoal. Finally the crystalline glutamic acid is obtained by adjusting the pH of the solution to its isoelectric point (pH 3.2) and separating the precipitate. A brief process flow sheet is shown in Figure 1.

b. Alkaline hydrolysis of protein rich compounds. Steffen's Molasses from a beet sugar plant is usually used as the starting material. The molasses is first saturated with carbon dioxide, and the solid impurities are removed by filtration. The clear filtrate is then concentrated. The precipitates produced during the concentration

are removed by sedimentation and filtration. The filtrate is concentrated again. The liquid is then hydrolyzed by alkali. By adjusting the pH of the hydrolyzate to 5.8 inorganic salts are salted out. After filtration, the hydrolyzate is cooled down to a temperature below $0^{o}C$ and the crude glutamic acid crystals are obtained. The amino acid can be further purified by neutralization, de-colorization and filtration. A brief process flow sheet is shown in Figure 2.

Microbiological fermentation

The usual culture medium for glutamic acid fermentation contains a carbon source such as glucose, the acid hydrolysate of starch, molasses, or a mixture of these substances. A nitrogen source such as urea, and other chemicals is present. The prepared culture medium is sterilized in a fermentor by steam. When the temperature of the medium cools down to $30^{o}C$, the micro-organism is added to the fermentor in a proper inoculum size. In this method, the micro-organism, Micrococcus glutamicus or some other glutamic acid producing micro-organisms are used. The micro-organism is incubated for thirty-six to forty-eight hours during which time the pH, temperature, and aeration rate are carefully controlled. When the fermentation is finished, the fermentation broth is hydrolyzed with hydrochloric acid. Glutamic acid is obtained in a process analogous to that for the recovery from the protein hydrolysate. The process flow sheet is shown in Figure 3. (Su 1963)

The synthetic method -

Since the beginning of the 19th century the synthetic methods of producing glutamic acid have been intensely studied by many investigators. Gresham and Schweitzer (1951), Schlemuth (1960), Gunthe and Schnell (1930), McIlwain and Richardson (1939), Keimatsu and Sugasawa (1925), Takagi and Haga (1961), have presented methods of producing glutamic acid from various raw materials by synthetic means. A stepwise procedure for producing glutamic acid by a synthetic method, which has been already applied in industrial production is shown in Figure 4.

Since our primary interest is in the microbiological production of glutamic acid a more detailed discussion of this method follows.

Glutamic Acid Fermentation

Micro-organism. In 1957 Dr. Kinoshita (1963) and Professor Asai (1959) (Tokyo University) reported that they had isolated from soil a strain of Micrococcus glutamicus, which was able to accumulate glutamic acid. Thirty per cent of the glucose consumed during the fermentation in a glucose-ammonium medium was converted to glutamic acid by this organism. Since then, a number of investigators have worked intensively in this field. They have found several bacteria and fungi which excrete appreciable quantities of glutamic

acid when grown on glucose-ammonium media. The best producers are: (Su 1963)

Table 4. List of Glutamic Acid Producing Micro-organism

Micro-organism	Investigator
Micrococcus glutamicus nov. sp.	Kinoshita (Kywa Fermentation Industry Co., Ltd.)
Brevibacterium divaricatum nov. sp.	Su, Yamada (Tokyo University)
Brevibacterium aminogenes nov. sp.	Tanaka (Shin Shin Food, Inc.)
Brevibacterium flavum nov. sp.	Okumura (Ajinomoto Co.)
Microbacterium ammoniaphilum	Miai

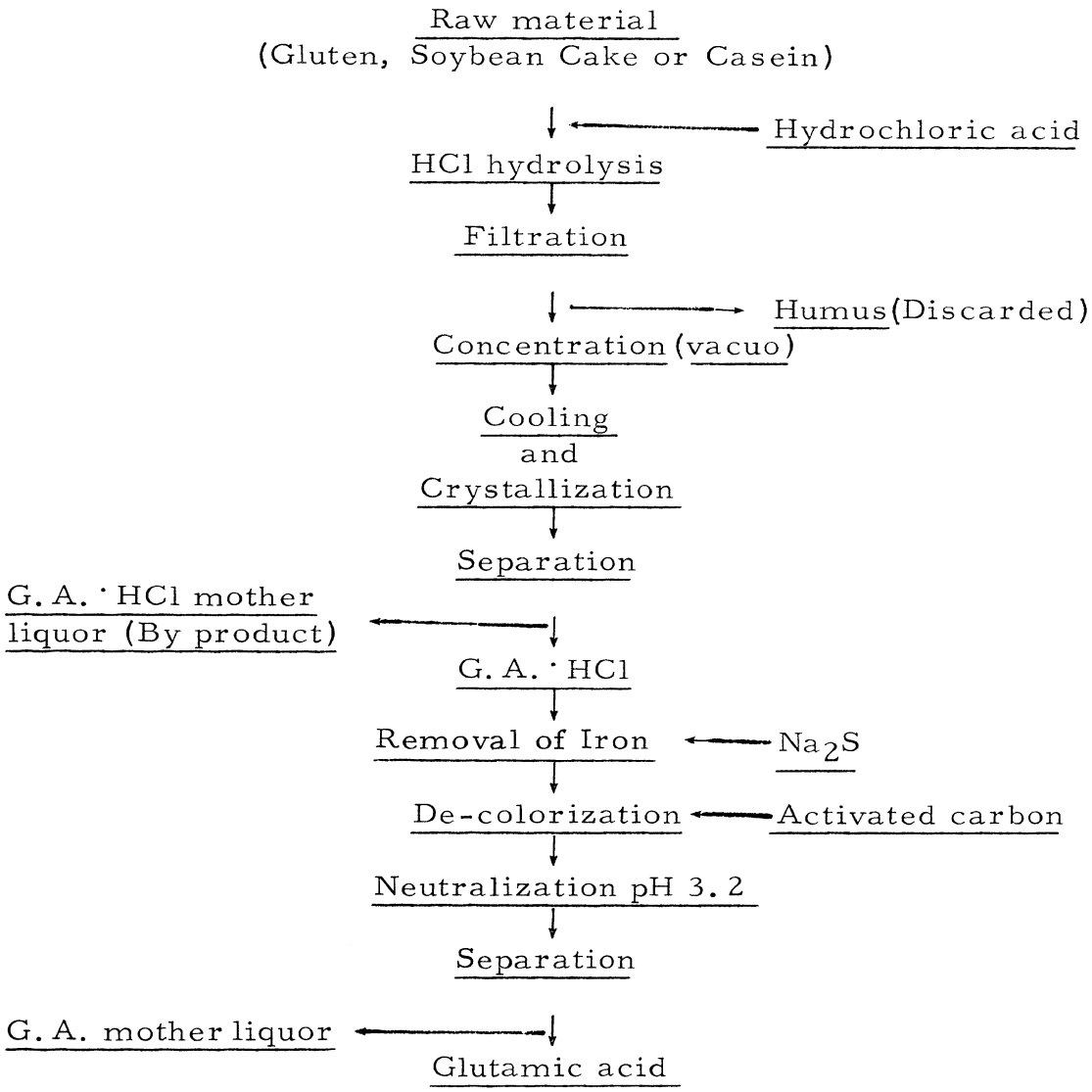


Fig. 1 Flow Sheet of HCl Hydrolysis of Protein Compounds

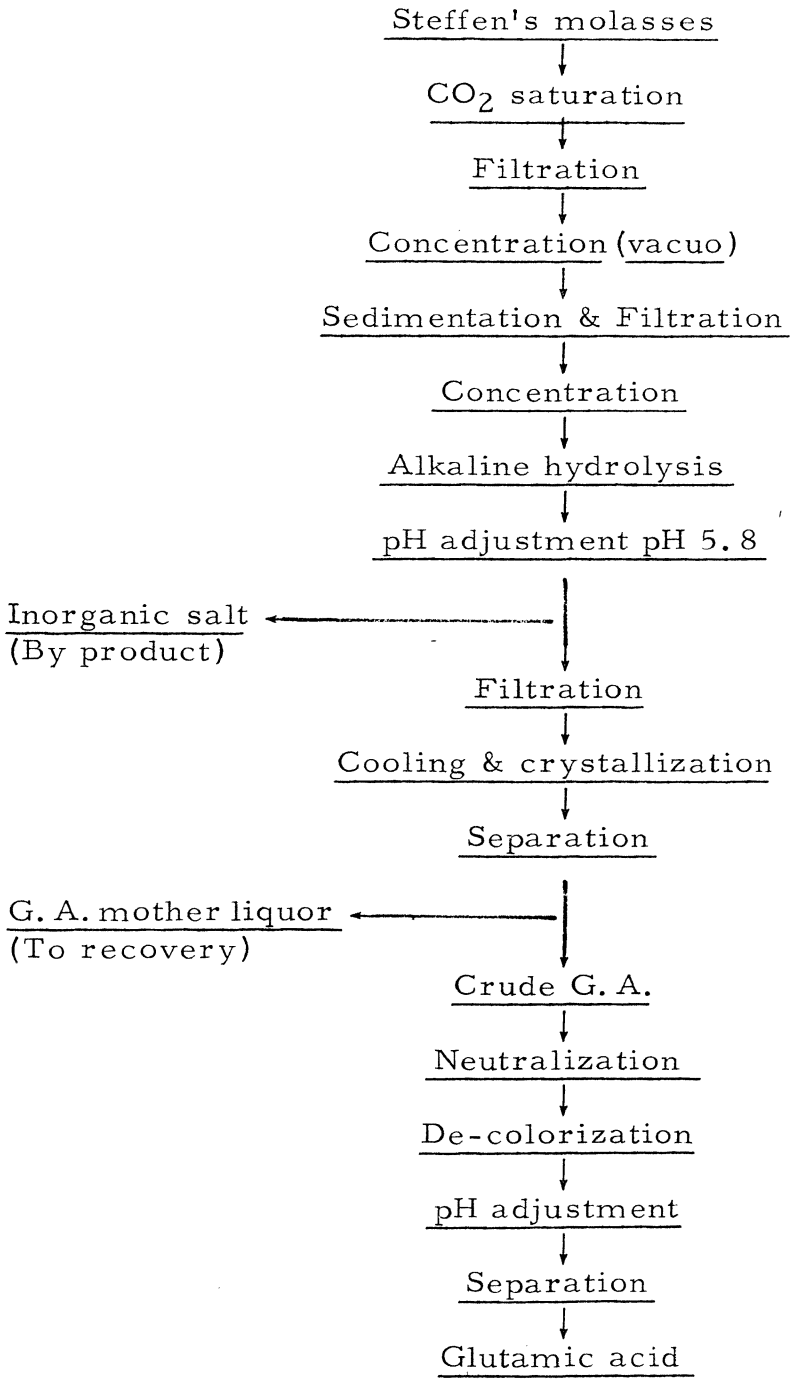


Fig. 2 Flow Sheet of Alkaline Hydrolysis of Steffen's Molasses

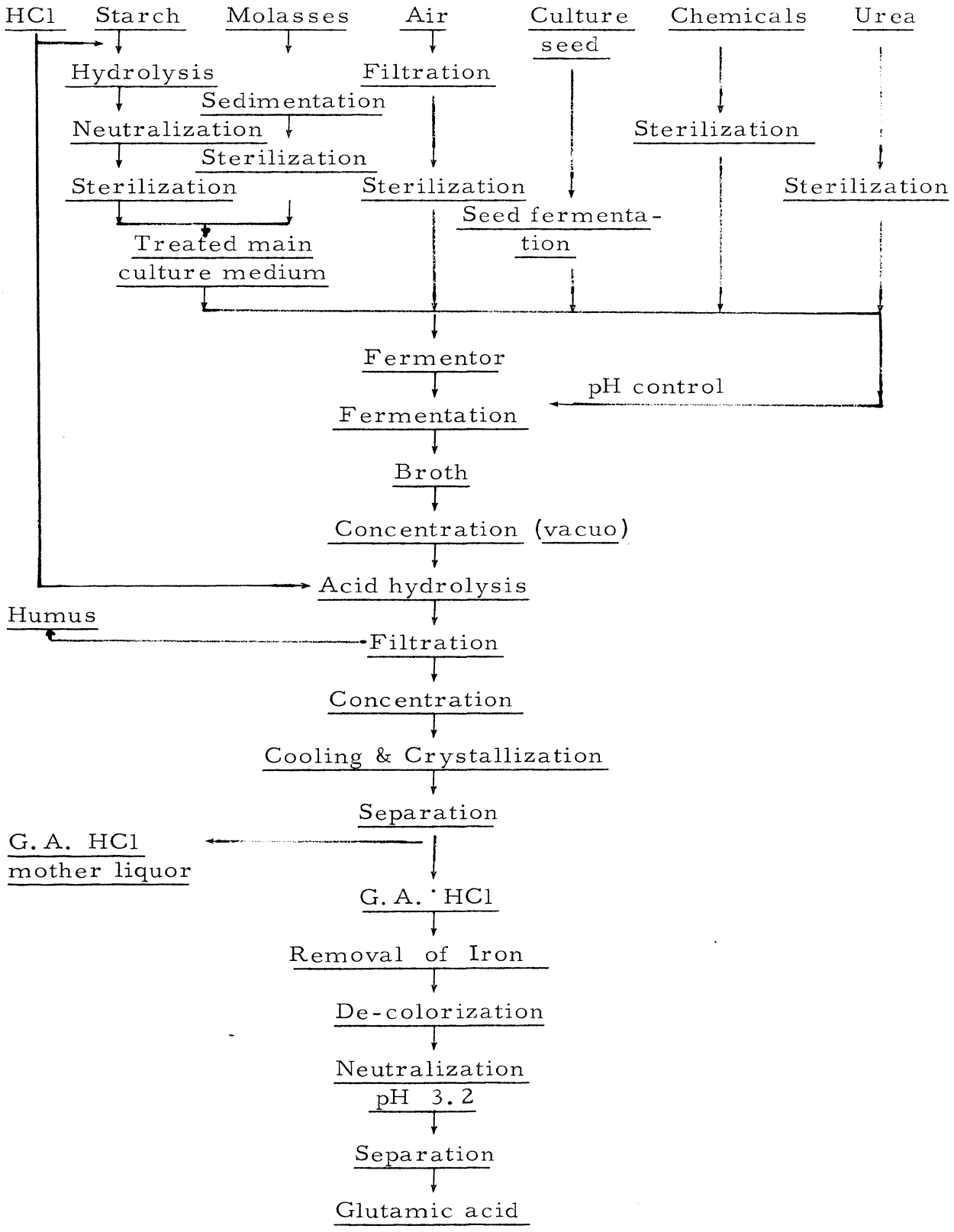


Fig. 3 Flow Sheet of the Fermentative Method of Glutamic Acid Production

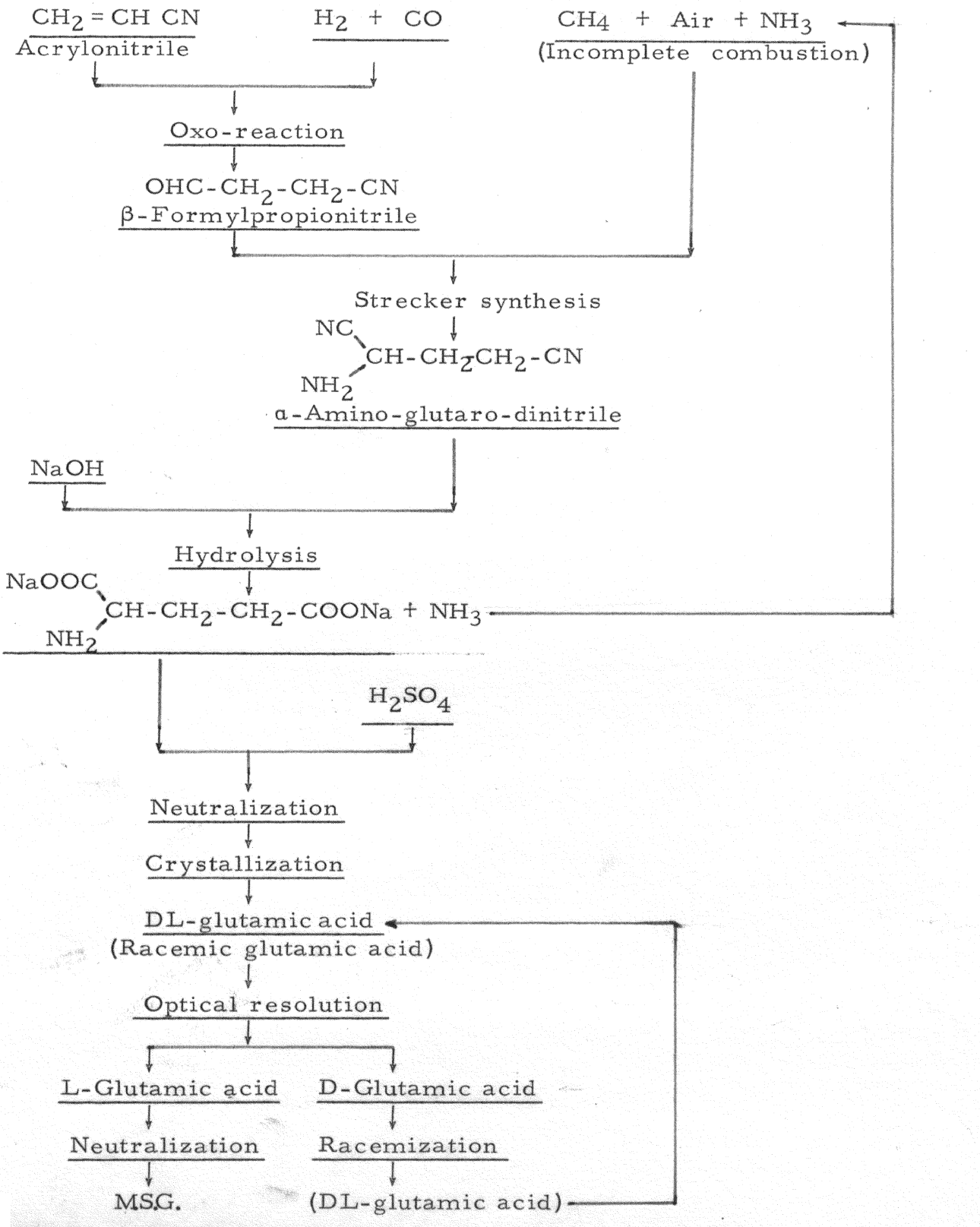


Fig. 4 Flow Sheet of the Synthetic Method of Glutamic Acid Production

Other organisms found to accumulate significant quantities of glutamic acid were; Corynebacterium lilium and C. callunae (International Minerals & Chemical Corp. 1962, Lee and Good 1962), Brevibacterium devaricatum (McCutchan and Hidy 1962), Br. lactofermentum (Motozaki, Okumura, Ishikura, Tsunoda, and Okada 1962), Br. saccharolyticum, Br. flayum, Br. immariophilium, Br. roseum (Okumura, Tsugawa, Tsunoda, Kono, Matsiu, and Miyachi 1962) and Bacillus megatherium (Ogawa, Tsubida, Okukura and Ozaki 1962).

Though these micro-organisms have some differences in physiological and morphological characters, most of the organisms, which are at least good glutamic acid producers, are found to have the following common characteristics; (Kinoshita et al., 1963).

1) Cell form is coccal to short rod.

2) Positive Gram stain.

3) Aerobic.

4) Nonsporulating.

5) Require biotin as an essential growth factor.

6) Accumulate large quantities of glutamate from carbohydrates and ammonium ions in aerated culture.

The morphological forms of these strains are somewhat variable depending upon the composition of the culture medium. One of the chief factors which affects cell form is the concentration of biotin in the medium (Kinoshita 1963).

Culture media. Various type of media have been used by investigators. In order to get a high yield of glutamic acid, some specially designed media are prepared for different strains of micro-organisms. The compositions may differ in certain aspects from each other, yet the main constituents of the media are the same. The media may be broken down into the following four categories: carbon source, nitrogen source, growth factors, and other chemicals.

Carbon source. Glucose and sucrose are the best carbon sources for glutamic acid producing micro-organisms. On the industrial scale production of glutamic acid, a hydrochloric acid or sulfuric acid hydrolysate of casava starch has been used in place of glucose. Some impurities, for instance, biotin in the crude sucrose can affect the production of glutamic acid (Ting and Lee, 1959). Molasses from a sugar plant may be used as a carbon source; but because of its rich biotin content, it needs special treatment before it can be used, or else some special controls during the fermentation are required (Crook 1964).

The yield of glutamic acid vs. the amount of glucose consumed, is greatest when the concentration is about 5% glucose, but an adequate sugar concentration is dependent on the nitrogen source. Though the percentage yield based on consumed glucose is maximum at 5% glucose concentration, for the purpose of increasing total

yield of glutamic acid in industrial practice 7-10% glucose is recommended. A higher concentration decreases the yield of glutamic acid based on glucose consumption (Ting 1959). It is believed that the organisms used in this process can produce over 50g of glutamate from 100 to 150g of glucose (Kinoshita et al., 1963). Glucose in the media plays a role as an energy supplier for the growth of the organism and the raw material for glutamic acid formation.

Nitrogen source. The best nitrogen sources are urea, $(NH_4)_2SO_4$ or NH_4Cl. The optimum concentration for urea is in the range of 0.5-1.0% for $(NH_4)_2SO_4$ 0.2-0.5% and for NH_4Cl 0.3% (wt.) (Ting 1959). An excessive amount of urea in the culture media tends to make the culture alkaline, and an excess of $(NH_4)_2SO_4$ causes the media to become acid. Combinations of these chemicals can be used. Urea solutions can be added during the fermentation as an additional nitrogen source and also as a pH controlling agent. If a considerable amount of ammonium ion is present in the medium, Micrococcus glutamicus will accumulate mainly glutamine, instead of glutamic acid (Crook 1964).

Biotin. As mentioned in the common characteristics of good glutamic acid producing organisms, biotin is a growth factor for these micro-organisms (Kinoshita et al., 1963). This vitamin plays a key role in the medium for glutamic acid accumulation. A high

content of biotin in the medium will promote rapid propagation of the micro-organism, but will produce little glutamic acid (Ting and Lee, 1959). High glutamic acid yields are always obtained in media containing a suboptimal concentration of biotin (Kinoshita et al., 1963). The best concentration of biotin in the culture medium is 2-3 γ/1. (Tanaka 1960). The deficiency of biotin affects the composition of the cell wall or the cell division mechanism. Biotin may also affect the physicochemical properties of the cell, such as the permeability of the cell wall (Kazuo Kimura 1963).

Other chemicals. A complete medium for glutamic acid fermentation contains, besides carbon, nitrogen and biotin, some inorganic salts. Inorganic salts, such as KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, are usually used in the culture media. Some of these salts effect the biosynthesis of the amino acid, and the others act as a buffer in the culture medium.

A typical composition of a medium is shown below (Kazuo Kimura 1963):

Table 5. Medium for Pre-culture

Glucose	2 g
Peptone	1 g
Meat extract	0.5 g
NaCl	0.25 g
H_2O	100 ml

Table 6. Fermentation Medium

Glucose	100.00 g
KH_2PO_4	1.00 g
K_2HPO_4	1.50 g
$MgSO_4 \cdot 7H_2O$	0.75 g
$FeSO_4 \cdot 7H_2O$	0.02 g
$MnSO_4 \cdot 4H_2O$	0.02 g
$(NH_4)_2 SO_4$	5.00 g
Biotin	2.50 γ
H_2O	1000 ml

Fermentation Conditions

Temperature, aeration pH, and foaming are the important variables which control the fermentation. Change in any of these conditions will change the whole process and the product of the fermentation.

Temperature. A suitable temperature range is required for growing a micro-organism in a culture medium. It is determined by the optimum temperature for reactions of enzymes in the cell. The cell growth will increase with the increase in the temperature of the culture medium. But, temperatures higher than 70°C will cause the denaturation of the enzymes and thus effects the yield of product. It is known that the optimum temperature for product formation is not the same as that at which the micro-organism grows most rapidly. The optimum temperature for producing glutamic acid by Micrococcus glutamicus is between 28-32°C (Tanaka 1960). Usually, the temperature of the fermentor is maintained at 30°C during the fermentation. Therefore, in order to maintain this

temperature, a heating and cooling system is required for a fermentor.

Aeration. Micro-organisms that are good glutamic acid producers are aerobic organisms (Kinoshita et al., 1963). In order to keep these micro-organisms growing and accumulating the amino acid and an adequate amount of air must be supplied. There is evidence which supports the fact that either under a restricted aerobic or an anaerobic condition, glutamic acid will not accumulate (Okada, Kameyama 1962). A balance of aerobic and anaerobic conditions is therefore one of the important factors which control the yields of glutamic acid. To get an effective oxygen diffusion rate in an Erlyenmeyer flask a shaker (rotary shaking 200-220 rmp) or an agitator and a sparger in a fermentor is required. The optimum value for the specific oxygen diffusion coefficient (K_d), or more commonly called a volumetric mass transfer coefficient, in an actively growing culture is reported to be in the range 3-5 x 10^{-6} g mole O_2/atm/min/ml. (Negishi 1959, Su and Yamada, 1960).

pH control. Glutamic acid producing micro-organisms prefer to grow in a slightly alkaline medium. The pH of the culture medium is usually held in a range between pH 7.0 to 7.8. During the fermentation, because of the utilization of ammonium ions, the pH of the medium drops gradually. In order to keep the pH at the optimum pH of the micro-organism, it is necessary to add urea

solution into the culture medium continuously. A pH measuring and controlling device is required for a fermentor. (Su 1961)

Foam control. Aeration and agitation of microbial cultures often causes foaming. This can be a serious problem in the microbiological industries, for foaming can lead to contamination and loss of material, either of which will reduce yields. Media containing molasses, yeast extract, peptone or corn steep liquor foam more readily because of the foam-stabilizing properties of the polypeptides in these materials (Rose 1961). Foaming is usually controlled by either mechanical methods or adding some form of non-toxic anti-foam to the cultures. The primary function of any defoaming agent is to reduce surface tension. Oils, particularly lard, soybean and linseed oils, are commonly used. Polyglycol and silicone oils are also used.

Dissolved Oxygen and Oxygen Determinat

As mentioned in the section of the fermentation conditions, aeration is very important in controlling a fermentation, and is especially so in the fermentation of glutamic acid by micro-organism. The balance between an aerobic and anaerobic condition will effect the yield of a fermentation (Kinoshita et al., 1963). It is believed that only the dissolved oxygen in the liquid phase of the medium is available to micro-organisms. The problem of supply and demand

of oxygen to the respiring cell becomes one of mass-transfer of oxygen molecules from the gaseous phase, through the culture fluid to the cell, and into the cell. The resistances involved in this transfer can be shown as Figure 5 (Bartholomew 1950).

where;

$1/k_1 \propto$ gas-flim resistance between the bulk of the gas and the gas-liquid interface;

$1/k_2 \propto$ gas-liquid interfacial resistance. (Only those oxygen molecules with sufficiently high energy can penetrate into the liquid, the remainder being reflected back into the gaseous phase);

$1/k_3 \propto$ liquid-film resistance extending from the gas-liquid interface to the bulk of the liquid;

$1/k_4 \propto$ liquid-path resistance. (This is not generally considered to be of importance since the concentration of oxygen in bulk of the liquid is usually assumed to be constant. This may be true only for adequately agitated systems);

$1/k_5 \propto$ liquid-film resistance around the cell or cell-clump;

$1/k_6 \propto$ intracellular or intraclump resistances, which are dependent on the type of growth obtained. This factor may assume some importance particularly in mold fermentations producing pellets or spheres of tangled hyphae;

$1/k_7 \propto$ reaction resistance. Resistance to the reaction of oxygen molecules with the cell respiratory enzymes.

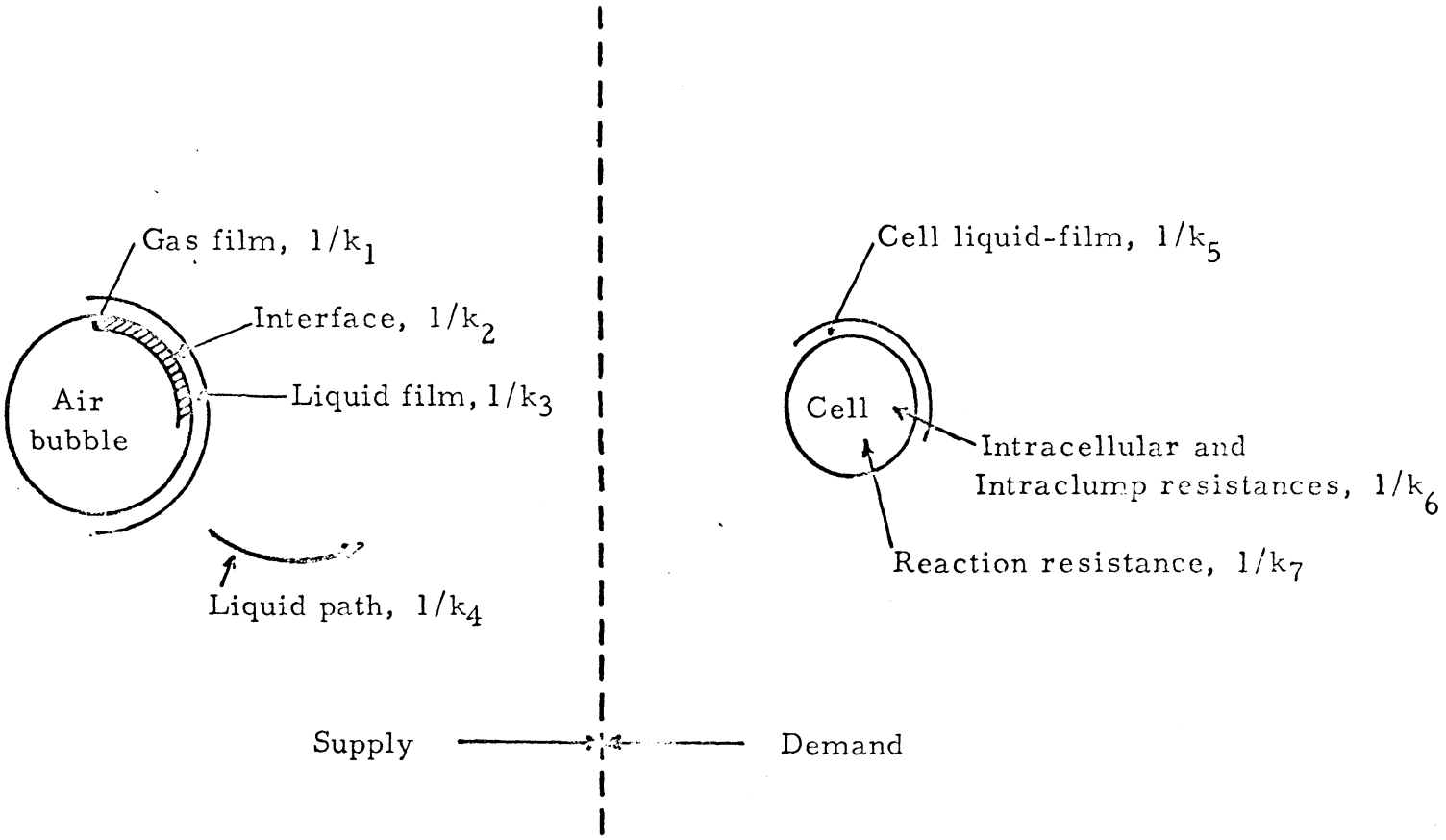


Figure 5. Resistances to Oxygen Transfer Form Gas Bubble into Microbial Cell

In practice it is extremely difficult to measure the concentration of oxygen at, for example, the interface between air and liquid or at the cell wall. Therefore some investigators have suggested ways to approach this transfer mechanism. The individual resistances are usually grouped together to permit consideration only of an oxygen concentration that can be measured readily. By means of the Whitman theory (1926) of gas absorption, an overall oxygen transfer coefficient can be evaluated. Because of the complexity of the evaluation of this coefficient, the evaluation is omitted here. However, the detailed evaluation can be found on page 162 of Biochemical Engineering, edited by R. Steel.

In the biochemical engineering field there is much interest in the oxygen uptake rate of micro-organisms. The oxygen uptake rate, the rate of the consumption of dissolved oxygen in the medium by micro-organism, is determined by the concentration of dissolved oxygen and the number or dry weight of the cells in a fermentative medium. The oxygen uptake rate can be expressed in the following equation; (Richards 1961)

R'_{O_2} = milli-moles of dissolved oxygen consumed/hour, micro-organism (or gram of dry weight of micro-organism)

In order to measure the concentration of the dissolved oxygen various techniques and analyzers have been suggested by many investigators.

The most widely accepted of the measurement methods are described as follows:

Sulfite method. (Cooper, Fernstrom, Miller 1944, Schultz 1956). This method of dissolved oxygen measurement is used in the determination of the rate of oxygen supply. A sulfite solution is aerated with air and a sample is periodically removed from the fermentor. The un-oxidized sulfite is determined iodometrically and the oxygen dissolved during the period is calculated. Because of the toxic effect of the sulfite ion on a biological system this method can not be applied for the measurement oxygen transfer in a fermentor containing micro-organisms.

Polarographic method. With a specially designed cell the dissolved oxygen concentration of a medium can be determined by the principles of polarography. However, it has been shown that mercury of the cells has toxic effect on biological systems, and there is adsorption of organic compounds on the platinum electrode. Therefore, this kind of oxygen analyzer has its limitations (Richards 1961).

Galvanic oxygen analyzer cell. This kind of analyzer is a design based on the principle of air cell. The detail of the oxygen analyzer was suggested by Mancy and Westgarth (1962) and is shown in Figure 6. A silver-lead galvanic half cell in 1 M KOH solution

is used in the electrode system. The galvanic cell is fitted to the tip of a plastic probe and is covered by a Teflon membrane which is permeable to oxygen and impermeable to most other substances. This membrane layer serves as a selective diffusion barrier separating the galvanic cell from the test solution. The leads of the electrodes are connected to a microammeter or the voltage drop across a suitable resistor can be measured on a recording potentiometer.

The cell reactions are assumed to be as follows:

Cathodic reaction:

$$O_2 + 2H_2O + 4e \rightarrow 4OH^-$$

Anodic reaction:

$$Pb + 4OH^- \rightarrow PbO_2^{--} + 2H_2O + 2e$$

The current generated from this probe is in proportion to the oxygen concentration in contact with the cathode. Therefore, the concentration of dissolved oxygen can be determined by measuring the generated current from the probe or the voltage across a suitable resistor.

In the author's experiment a galvanic oxygen analyzer of the type described above was used.

Glutamic acid biosynthesis

The mechanism of the biosynthesis of glutamic acid from glucose-ammonium media by Micrococcus glutamicus has been studied during the last few years. Tanaka, Aida, Shiio, Yada, Okada (Kinoshita 1963) and Su (1961) reported their studies on the glycolysis steps of glutamic acid formation by the glutamic acid formation by the glutamic acid producing micro-organisms. It was confirmed that these organisms use both the Embden-Meyerhof-Parnas pathway and the Hexose Monophosphate Shunt. Figure 7 shows the possible biosynthetic pathway. The extent to which the Hexose Monophosphate Shunt pathway is used varies with the cultural conditions.

Using cell free preparations, glutamic acid is obtained quantitatively from citrate or isocitrate under anaerobic conditions. If ammonium ion is omitted from the reaction mixture, α-ketoglutarate and succinate are the main oxidation products. If the reaction is conducted under aerobic conditions, the recovery of these products decreases. Starting from glucose, glutamate is produced only under aerobic condition. Under anaerobic conditions lactate is formed. This evidence supports the assumption that the main path of glutamate synthesis involves a combination of oxidative degradation of glucose and anaerobic citrate decomposition.

It has been shown that the two NADP-specific dehydrogenases

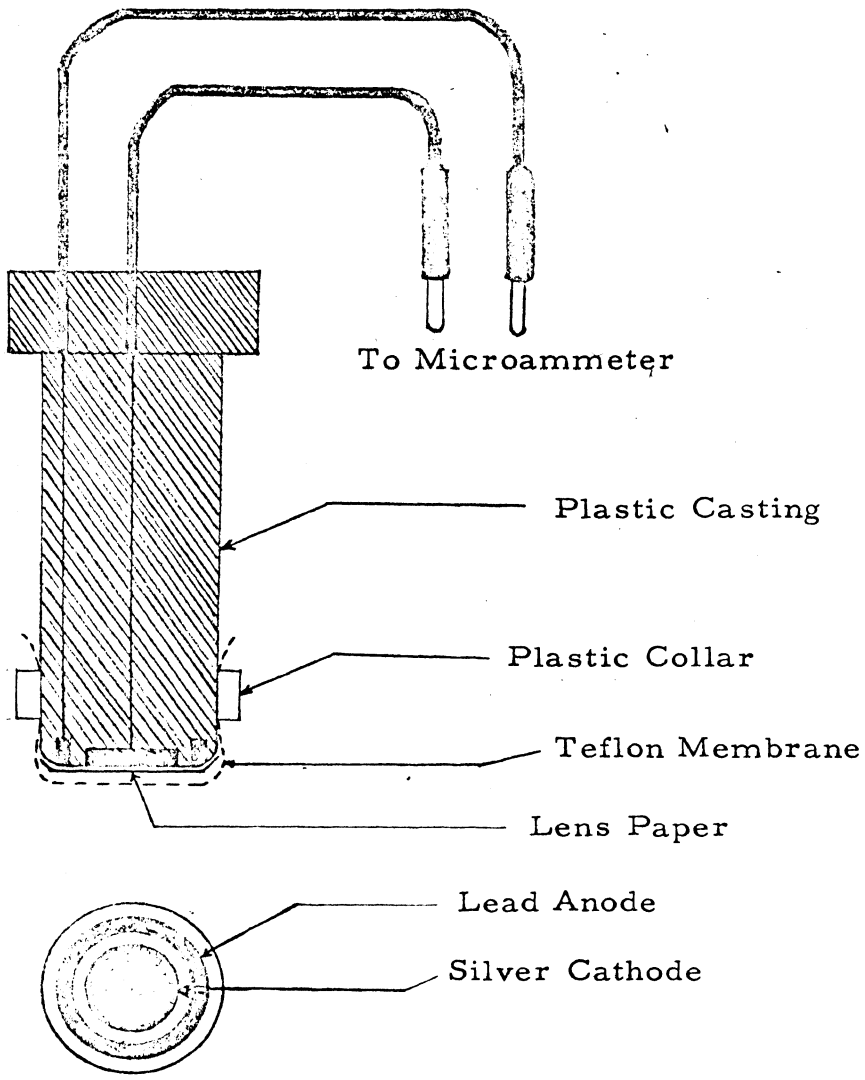


Figure 6. The Galvanic Oxygen Analyzer Cell

in Micrococcus glutamicus, i.e. isocitrate and L-glutamate dehydrogenase, are closely coupled in the presence of ammonium ions so that the oxidation product is trapped in the form of L-glutamic acid (Kinoshita 1957). Resting cells, incubated 24 to 48 hours, do not decompose or consume a glutamate substrate. This means the glutamic acid is one of the final fermentative products of this microorganism (Su, Tanaka and Yamada 1961).

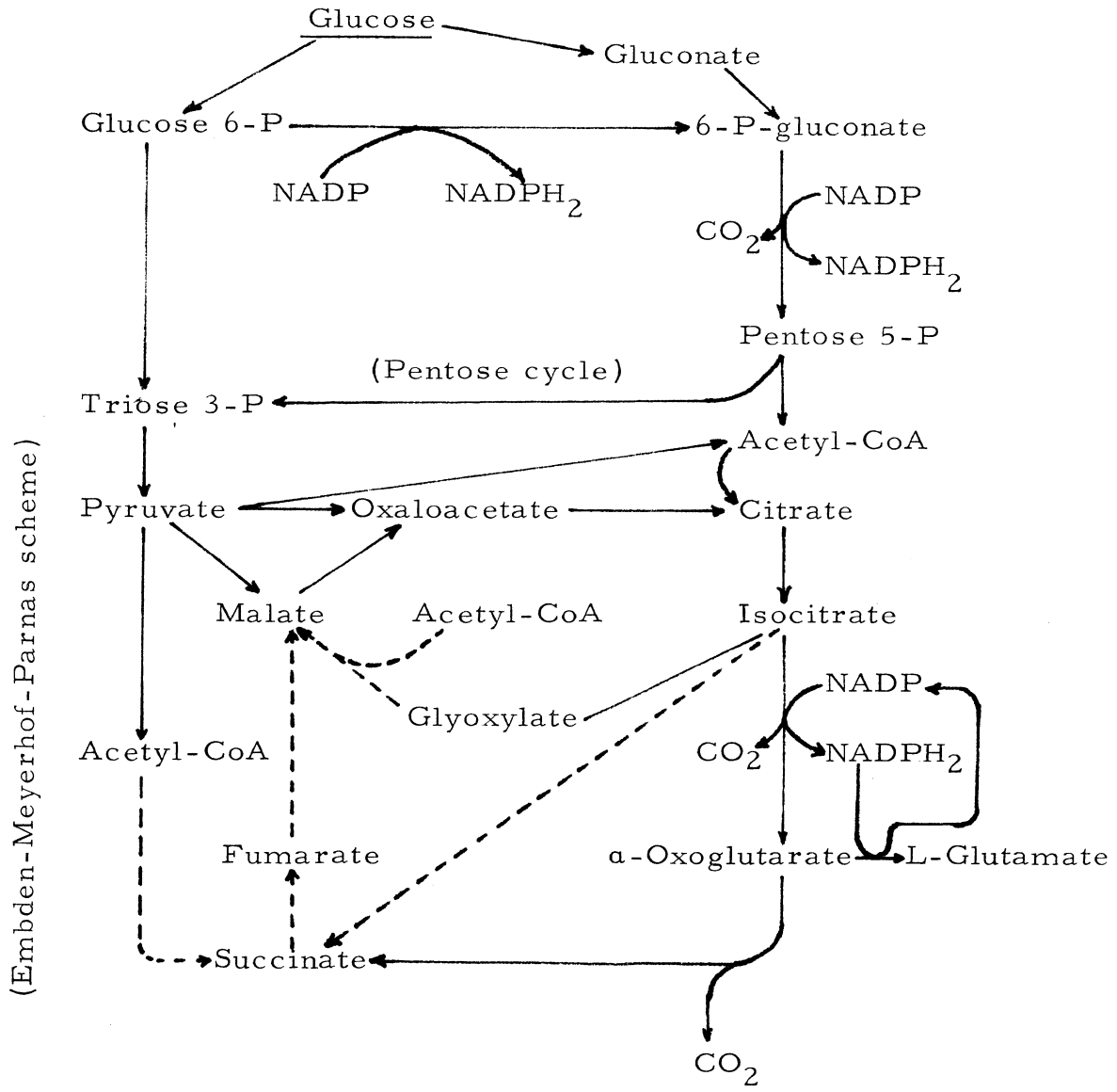


Figure 7. Probable Pathway of Glutamic Acid Formation by Micrococcus glutamicus

Assay Methods

In order to study the relation between glucose consumption and glutamic acid accumulation a complete set of assay methods was established. In the fermentation, glutamic acid is not the only amino acid produced in the fermentative broth. Sometimes, glutamine and other amino acids also appeared in the broth. Therefore, in order to detect what kind and amount of amino acids produced, both qualitative and quantitative methods of determination are required.

Qualitative method for amino acid determination. There are many methods for the determination of amino acids, such as, the Xanthoproteic, Molsch, and ninhydrin reactions. However, the most convenient and precise qualitative method of amino acid determination is paper chromatography. The general technique of paper chromatography method can be described as follows. Samples are first spotted on a piece of filterpaper. The paper is then placed in a solvent system. Because of the distribution between two phases (partition), adsorption and ion exchange of the unknown compounds the solvent system and the paper, the compounds are separated. When the individual compounds are made visible by a specific reagent, such as ninhydrin, the R_f values (the ratio between distance an individual spot moved and the distance the solvent front moved from the starting point) can be calculated. The R_f value is a characteristic of the amino acid, and by comparing the R_f values of known compounds

run under the same conditions with the R_f values of the unknown, the identity of the unknown can be determined. (Clayton and Strong, 1954)

Quantitative method for glutamic acid determination. Amino acid can be quantitatively determined by spectrophotometric or infrared methods. However the manometric method is the easiest way to determine glutamic acid. The principle of this method is the volumetric determination of carbon-dioxide produced from glutamic acid when the acid is reacated with an enzyme, glutamic acid decarboxylase. Because the carbon dioxide production is proportional to the glutamic acid present in the sample, the concentration of glutamic acid can be determined by the gas volume. In order to measure the gas volume a Warburg Respirometer is usually used. (Deluca and Cohen 1963)

Somogyi Micro-Copper method for glucose determination. The glucose determination has been studied by Shaffer, Hartmann, Somogyi (Hodge, Hoferiter 1962) and other investigators. They have developed several methods for the determination of reducing sugars. The copper reagents are more specific for sugars than ferricyanide or hypoiodite and, therefore, are widely preferred for analysis of biological material. The copper solutions used by various investigators may differ from each other, but the main reactions are the same. The cupric ion is first reduced by glucose to cuprous ion, and then the cuprous ion is reoxidized by iodine and the residual

iodine is determined by an iodimetric titration. Equations for the iodimetric reactions are;

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + H_2O$$

$$Cu_2O + 2H^+ + I_2 \rightarrow 2Cu^{++} + 2I^- + H_2O$$

$$I_2 + 2S_2O_3^{--} \rightarrow 2I^- + S_4O_6$$

III. EXPERIMENTAL

Material

Glucose (anhydrous grade III), L-Glutamic acid, L-Glutamic acid de-carboxylase, glutamine were obtained from Sigma Chemical Co., St. Louis, Missouri. Agar, peptone, casamino acid were obtained from the Difco Laboratories, Detroit 1, Michigan. Propionic acid (Assay 99%) was obtained from Matheson Coleman & Bell, Division of the Matheson Company, Inc. Methyl-ethyl-ketone used as the solvent system for the paper chromatography was reagent grade and was used without purification. Four types of antifoam agents were used. Antifoam AF 3, AF 10, and AF 20 (silicon antifoams) were obtained from General Electric Silicone Products Department, Waterford, New York. Another antifoam, Sag 470 Silicone Antifoam Emulsion was obtained from the Silicone Division, Union Carbide Corporation. Whatman's No. 1 Chromatography paper was obtained from Aloe Scientific Co., St. Louis, Missouri.

Micrococcus glutamicus (B. 2784) was obtained from Northern Regional Research and Utilization Laboratory, U.S. Department of Agriculture, Peoria, Illinois, NRRL and Micrococcus glutamicus T. was obtained from Wei-Chaun Foods Co., Taiwan, Republic of China.

Apparatus

Fermentor. A 5 liter fermentor, designed by Mr. Robert M. Logan (1963), was constructed by the author. A schematic diagram of the fermentor is shown in Figure 8., 9.

Oxygen Analyzer. The oxygen analyzing probe was constructed by Mr. Carl Wallace. The details of the design are shown in Figure 7.

Warburg Resperiometer.

Gilson Medical Electronics, Middleton, Wisconsin.

Incubator. The Electric Hotpack Company, Inc.

pH Electrodes.

Glass Electrode: Leeds and Northrup Company, No. 117123

Calomel Reference Electrode: Leeds and Northrup Company No. 117143

pH Indicator. Leeds and Northrup Co., No. 7664

Recorder. 12 Point Barber-Colman Co., Rockford, Illinois.

Pump (for antifoam).

Model AL 2.15, Sigmamotor Inc., Middleport, N. Y.

Solinoid Valve.

Catalog No. S90A1406 Hoke Inc., Cresskill, N.J.

Electronic Relay (Antifoam Control).

Herbach & Rademan, Inc., Philadelphia, Pennsylvania.

Rotameter (0-16 CFH). The Matheson Co., Inc.

Driving Motor (for agitator). General Electric Model 5KH38PG242E

Heating Element. Glas-Col Apparatus. Co Apparatus Co.

Shaker. Rotary type, 240 rpm., New Brunswick Scientific Co., New Brunswick, N.J.

Sterilizer. Rectangular Type, 24" x 36" x 48", No. 25870, American Sterilizer Co., Erie, Pennsylvania

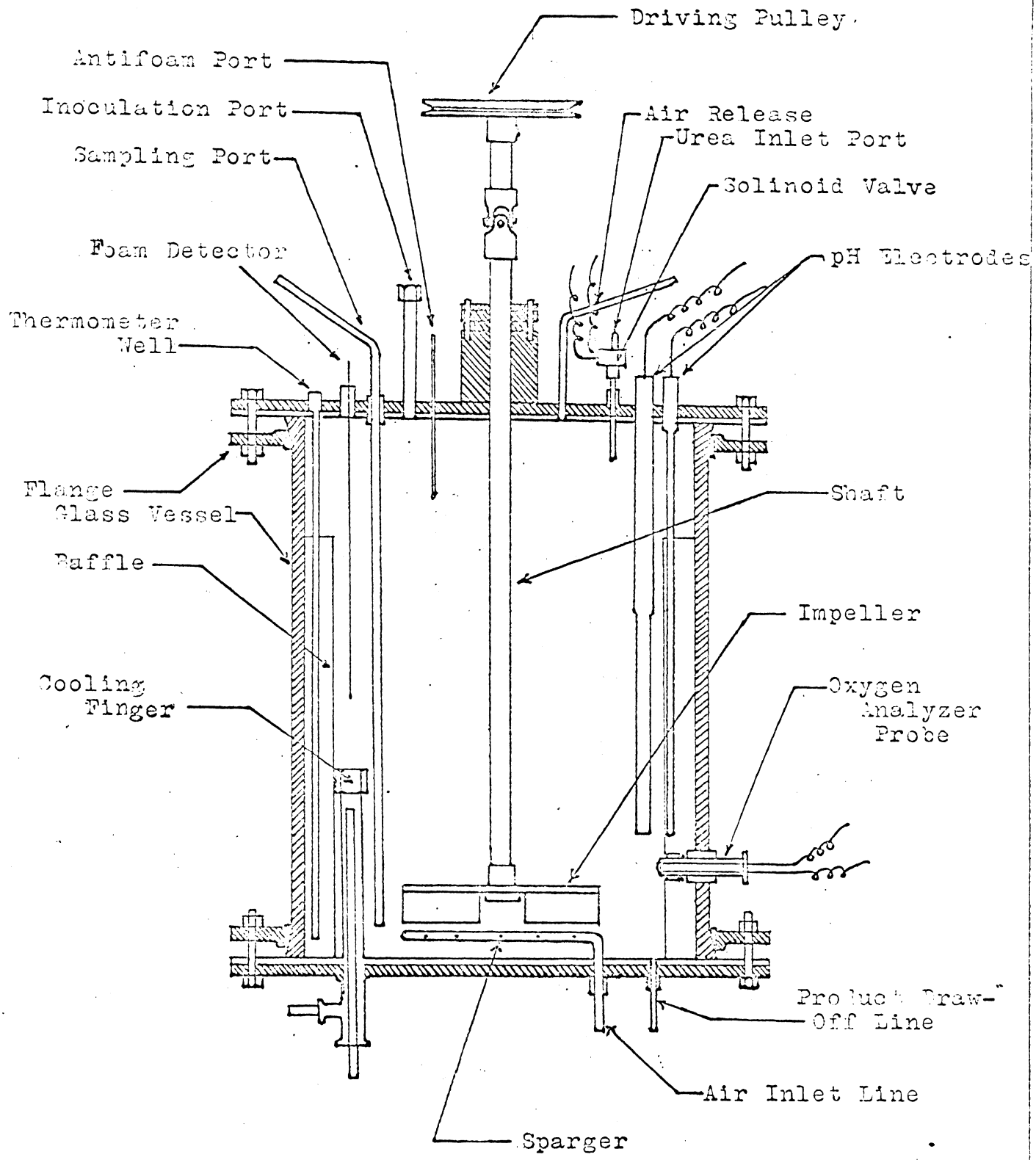


Figure 8. Schematic Drawing of 5-l Fermentor

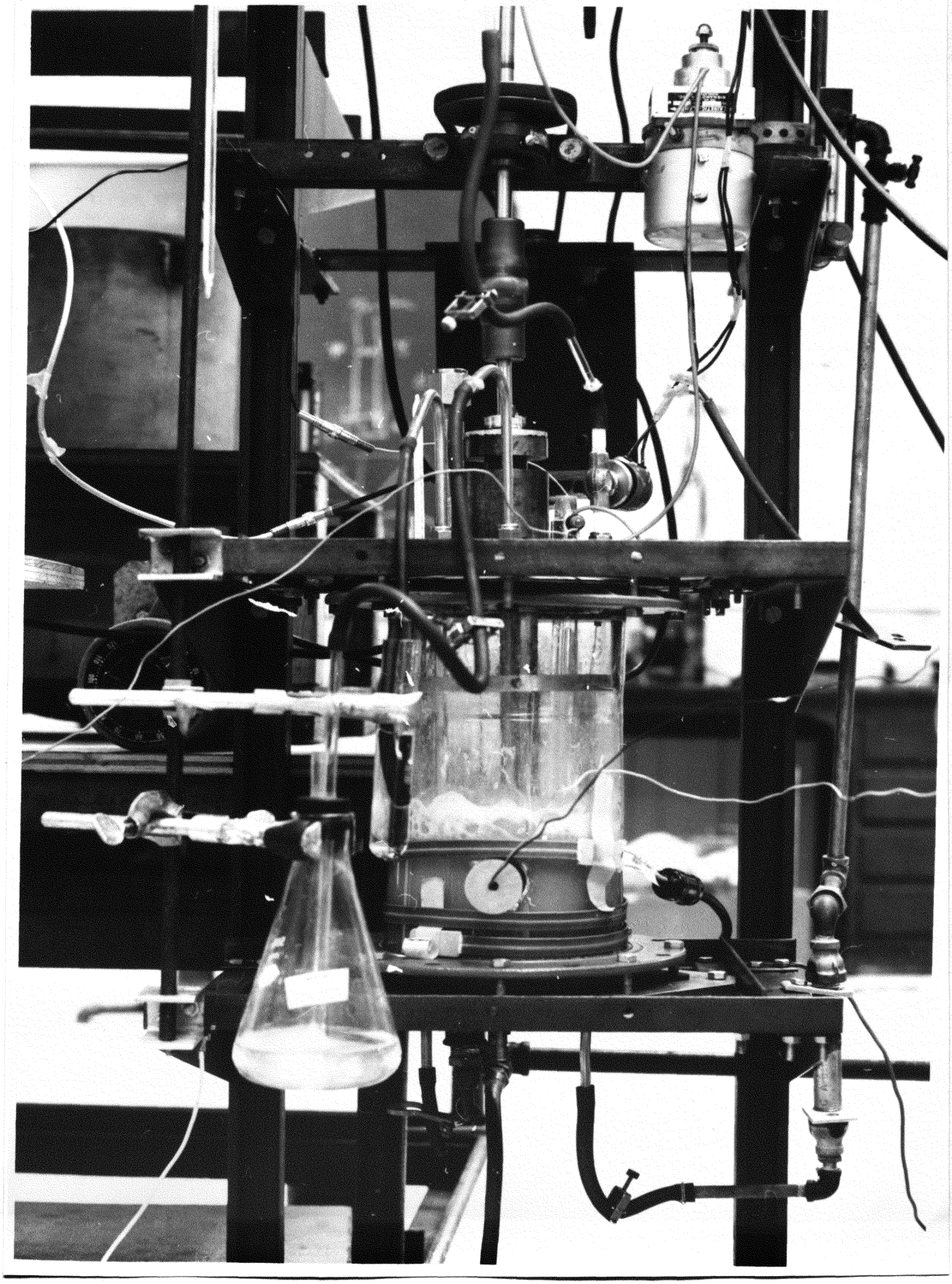
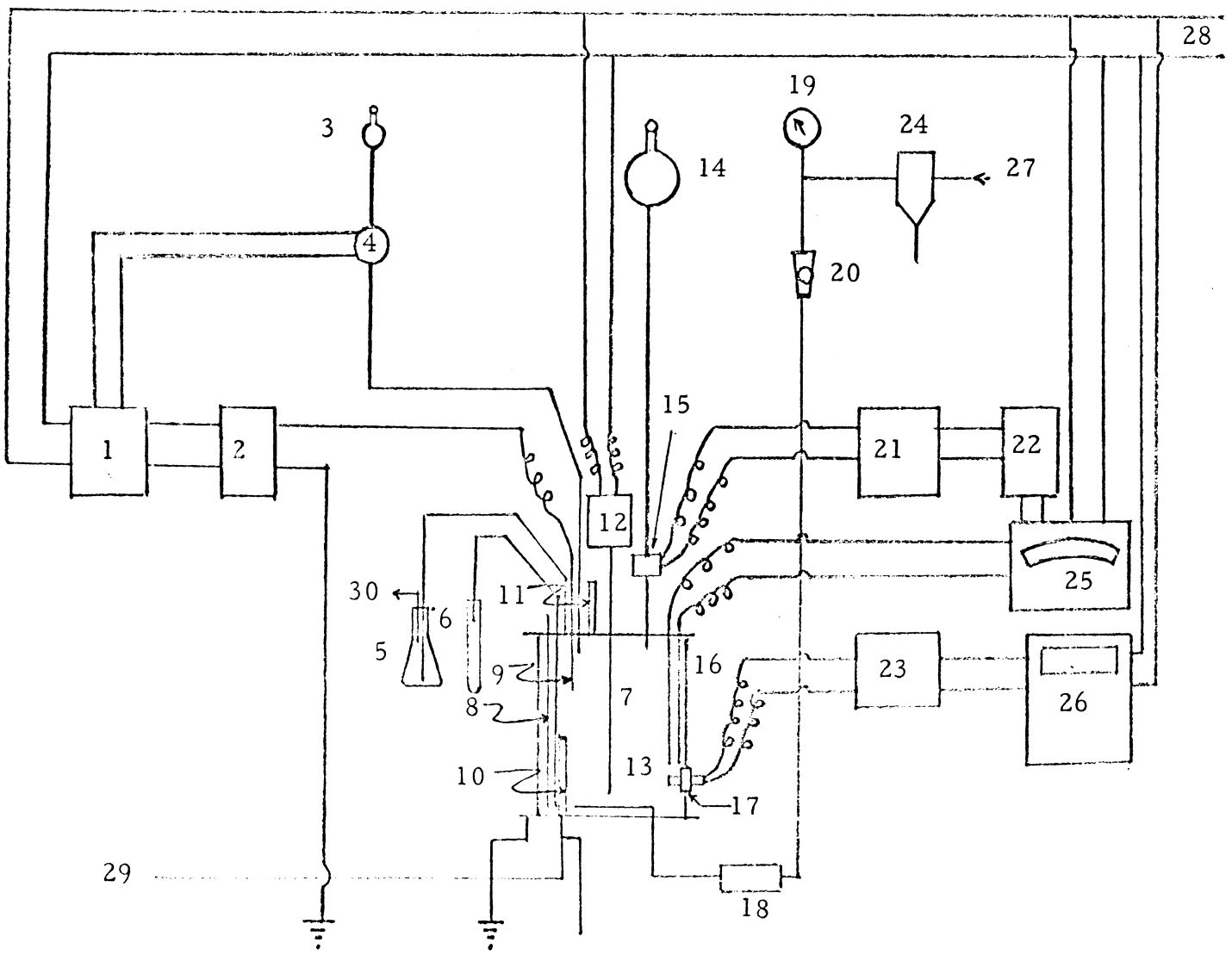


Figure 9. Photograph of 5-1 Fermentor



1. Time switch
2. Electronic relay
3. Antifoam reservoir
4. Pump
5. Ethanol sealing bottle
6. Sampling tube
7. Fermentor vessel
8. Thermometer
9. Foam detector
10. Cooling finger
11. Inoculating port
12. Driving motor
13. Impeller
14. Urea reservoir
15. Solinoid valve
16. pH electrodes
17. Oxygen analyzer
18. Air filter
19. Pressure gauge
20. Rotameter
21. Time switch
22. pH controller
23. Potential meter
24. Air trap
25. pH indicator
26. Recorder
27. Compressed Air
28. Power line
29. Water line
30. To CO_2 analyzer

Figure 10. Fermentor and Controllers

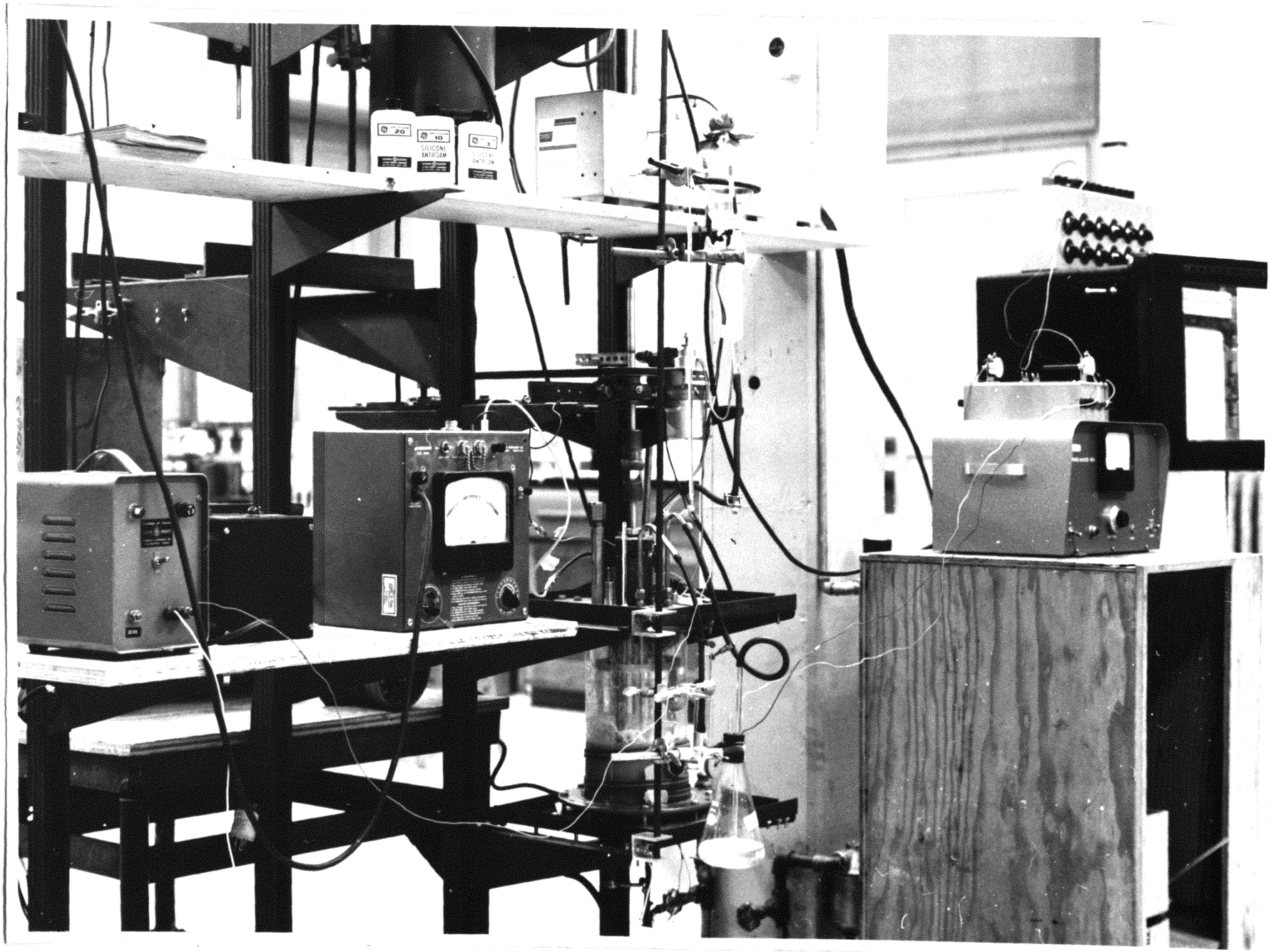


Figure 11. Photograph of 5-1 Fermentor Assembly

Cultivation of the Micro-organism

Storing of the Micro-organism and Seed Culture Preparation

Slants with the composition as shown in Table 7 were prepared and sterilized. The slants were all kept in a cold room (5°C) before use.

Table 7. Composition of Medium for Slants

Glucose	2.00 g
Peptone	1.00 g
Casamino Acid	0.20 g
NaCl	0.25 g
Agar	3.00 g
H_2O	100.00 ml

Each test tube contained 15 ml of medium.

The Micrococcus glutamicus strains which were obtained from the NRRL and Wei Chaun Foods Co., Taiwan (China) were transferred to the surface of the slants by means of a sterilized platinum wire loop. These slants were incubated at 32°C for 24 hours. At the end of the incubation, pale yellow colonies appeared on the surfaces of the slants. The incubated slants were kept in the cold room (5°C).

A seed culture had to be prepared before each fermentation, so that the micro-organisms would grow rapidly when the fermentation was performed. For a seed culture, one loopful of Micrococcus glutamicus growing on a slant was transferred into a 500 ml flask

containing 100 ml of seed culture medium.

Table 8. Composition of the Seed Culture Medium

Glucose	2.00 g
Peptone	1.00 g
Casamino Acid	0.20 g
NaCl	0.25 g
H_2O	100 ml

The pH was adjusted to 7.8 and the medium was sterilized at 250°F for 20 min.

The inoculated flask was incubated on a rotary shaker (240 rpm) at 26°C for 16 to 20 hours. This culture was used as the seed for the 500 ml shake flask and five liter fermentor fermentations.

500 ml Shake Flask Fermentation

By means of a sterilized pipet, ten milliters of the seed culture were transferred to a medium with the following composition.

Table 9. Composition of 500 ml Shake Flask Fermentation Media

KH_2PO_4	1.00 g
K_2HPO_4	1.50 g
$MgSO_4 \cdot 7H_2O$	0.75 g
$MnSO_4 \cdot 4H_2O$	0.02 g
$FeSO_4 \cdot 7H_2O$	0.02 g
Glucose	100.00 g
Urea	5.00 g
$(NH_4)2\ SO_4$	2.00 g
Biotin	1.25, 2.5, 10 γ
H_2O	1,000 ml
pH	7.8

Each flask contained 100 ml of the medium sterilized at 250°F for 20 minutes.

The inoculated flasks were incubated on a rotary shaker (240 rpm.) at 26°C ± 2°C for 48 hours. During the growth of cells the pH was controlled between 7.8 and 8.0 by the addition of 25% urea solution. At intervals of 6 to 8 hours 10 ml samples were taken from each flask. The pH, glucose and glutamic concentration of the samples were determined by the procedures described in page 51.

Five-liter Fermentor Fermentation

Before the medium was prepared in the fermentor, the product draw-off line and air inlet line, both at the bottom of the fermentor, were closed with clamps. The oxygen analyzing probe hold was closed with a rubber stopper. Two liters of medium of the same composition as used in the 500 ml shake flasks were added to the 5-liter fermentor.

The pH electrodes were standardized by using external electrodes before the pH of the medium was adjusted. The agitator was turned on and sodium hydroxide or sulfuric acid was added to the medium to adjust the pH to 7.8 to 8.0.

When the medium was ready to be sterilized, the rubber tubes, for transporting antifoam and urea solution from the antifoam and urea reservoirs, were connected to the fermentor. The reservoirs were filled with antifoam and urea solutions. The

sampling and air outlet tubes were closed by clamps. The ends of the inlets and air filter (Logan 1963) were plugged with cotton and wrapped with pieces of protective paper. The whole fermentor, including the air filter, was supported on a rack and put into an autoclave. The medium was sterilized at 250°F for 20 minutes.

After the sterilization, the fermentor was mounted on the frame (See Figure 9, 11) and the agitator was connected to a driving motor by a V-belt. Rubber tubing was attached to the cooling finger to carry water to and from the unit. In order to prevent possible contamination during the cooling period from outside of the fermentor, the aeration was started at the beginning of the cooling period. Thus there was always a positive pressure in the fermentor. The end of the sampling tube and the air outlet tube were each immersed in a test tube containing ethanol. The clamp on the air outlet tube was released as the aeration was started. When the temperature of the medium had cooled down (30-32°C), the pH electrodes were re-standardized using external electrodes.

During the cooling of the medium, the oxygen analyzer was prepared. The electrode surfaces of the analyzer were first carefully cleaned with distilled water and 1 M potassium hydroxide solution, then the electrodes were dried with clean tissue. A piece of lens tissue was placed on the electrodes with the analyzer facing

upward. The lens tissue was soaked with 1 M KOH solution. A teflon membrane (1/100 inch in thickness) was placed on the top of lens tissue and fitted firmly around the tip of the analyzer by means of the plastic collar (see Figure 7). It was important to make certain that the membrane was tightly fixed to the tip of the oxygen probe, and all air bubbles in the KOH solution were excluded. The excess membrane was cut above the collar. The probe was dipped in a sulfite solution after attaching the membrane, to allow the dissolved oxygen in the KOH solution to be consumed.

When the sterilized fermentor had cooled to 30-32°C, the fermentor was removed from the frame and held in such a position so that the medium in the vessel would not splash out when the rubber seal on the oxygen probe mounting hole was removed. The oxygen probe was taken from the sulfite solution and sterilized with ethanol. It was then inserted in the fermentor. The leads of the electrodes of the oxygen probe were connected to a potentiometer and a recorder. The resistance of the potentiometer was adjusted so that the reading of the recorder was about 0.9-0.95 of the full scale (10 millivolts) when the medium was saturated with oxygen.

When the analyzer had been set, the tube for transporting antifoam was attached to the Sigmamotor pump. The end of the foam detector on the top plate of the fermentor was connected to

an electronic relay and then to a time switch. The power output of the switch was attached to the pump. The foam control system was checked by grounding the detector to the fermentor frame. If the pump worked when the detector was grounded, the system was considered to be working properly.

After the controlling system and the measuring devices had been set, the fermentation was started by inoculating the fermentor with a 10% by volume inoculum of Micrococcus glutamicus seed culture. The inoculum was added through the inoculation port from the flasks. During the inoculation the mouths of the flask and the port were isolated from the open air by a piece of ethanol wetted cloth.

The fermentation was carried out under constant aeration, agitation and temperature. However, when it was necessary to change these variables, they could be adjusted by the following procedures. The rate of aeration was changed by controlling the needle valve of the rotameter on the air inlet line. The temperature was controlled by changing the rate of the water running through the cooling finger or by changing the voltage of the heating element wound around the fermentor. The rotational speed of the impeller was changed by changing the diameter of the pulley mounted on the agitator shaft.

During the fermentation, temperature, aeration and pH of the medium were recorded and checked from time to time. Samples were taken periodically from the fermentor. The samples were kept in test tubes and the glucose concentration and glutamic acid concentration were determined by the methods described below.

For the purpose of studying the relation between oxygen uptake and the growth of the organisms during the fermentation the rate of oxygen consumption and the cell number were determined. One hour after the fermentor was inoculated the aeration was stopped by closing the inlet air valve. Because of the uptake of the dissolved oxygen by the organisms, the oxygen concentration inthe medium was gradually decreased until all the dissolved oxygen in the medium was removed (1 minute to 30 minutes), The decrease of the oxygen concentration was recorded by the recorder. By measuring the slope of the oxygen concentration curve on the recorder chart, the rate of oxygen change was determined. The same tests were repeated every two to three hours.

The cell number was determined by the plating method. An estimation of the cell concentration in a sample taken from the fermentor at the time of the oxygen consumption test was made. The sample was then diluted with sterilized water until a one milliliter aliquot was thought (by experience) to contain from 30 to 300 cells.

The aliquot was then pipetted into a test tube containing 15 ml of warm (about 45-50°C) agar medium with the same composition used for the storage of cultures of Micrococcus glutamicus. The sample and agar solution were mixed by gently swirling to evenly disperse the cells throughout the agar medium. The mixed solution was poured into a sterile petri dish, and the agar was allowed to solidify. When the agar was solid, the dish was placed in an incubator at 32°C until visible colonies developed from the individual cells.

If the sample was properly diluted, the total number of the colonies on a plate could be directly counted. However, in some cases the number of the colonies were too high to be easily counted. In these cases a piece of paper divided into one cm squares was placed under the dish and the colonies were selected in random squares, at least one fourth of the total area of the dish was counted. The average number of the colonies in a one cm square was calculated. The total number of colonies in a sample was obtained by multiplying the average number of colonies per square by the total number of squares contained in a petri dish.

Analytical Procedures

Qualitative Analysis of Amino Acids by Paper Chromatography

Samples of fermentation broth, already dilute to the proper concentration, were spotted on a sheet of Whatman's No. 1 filter

paper (11 x 11 inches) with a 10 λ micro-pipet. For the purpose of comparison, a solution of known amino acids was also spotted on the paper. The spots were placed one inch apart on a line 2 cm above the bottom edge of the paper.

The paper was folded to form a cylinder, and stood in a jar containing the solvent system, (about 1 cm deep) methylethylketone-propionic acid-water (75/25/30 ratio by volume). A glass plate was placed on the jar and the jar was sealed with modeling clay. When the solvent front had moved about 8 inches above the bottom of the paper, the paper was taken out of the jar, the solvent front was marked with a pencil and the paper was dried in air. After the paper was dried, it was sprayed with a ninhydrin solution (0.2% ninhydrin dissolved in n-butanol to which was added 5 mg phenol per 100 ml of solution). The color of the individual spots appeared when the paper was dry. In order to preserve the color of the developed spots, the paper was sprayed with a copper nitrate solution (1 ml saturated aqueous copper nitrate in 100 ml ethanol plus 0.2 ml 10% HNO_3). The R_f value of each spot was calculated by measuring the distance the spot and solvent front moved from the starting line. The amino acids were determined by comparison of R_f to that of known compounds.

Manometric Method for the Determination of Glutamic Acid

The enzyme solution used in this determination was prepared

by suspending 15 mg of L-glutamic acid decarboxylase in one ml of 0.1 M acetate buffer. The solutions to be analyzed for glutamic acid were brought to a pH of 5 adding 3 M acetate buffer prepared by dissolving 27.2 g of sodium acetate plus 6 g of glacial acetic acid in water and diluting the solution to 100 ml with distilled water. The samples were diluted to a concentration sufficient to yield a measurable gas production in the manometer. One milliliter of this solution and one milliliter of 0.1 M acetate buffer solution were added to the main compartment of a manometric flask. 0.5 ml of enzyme solution was added to the side arm. The flask was attached to a manometer and immersed in a constant temperature water bath (37°C). The flask assembly was continuously shaken in the water bath for about ten minutes to reach temperature equilibrium. When the flask contents had reached the desired temperature, the right arm of the manometric liquid was adjusted to the 150 mm level. The gas releasing cock was closed, and the flask contents were mixed by inclining the flask assembly. When the end point of the reaction was reached, the level of the manometric liquid was readjusted to the point where it was set at the beginning of the reaction. By reading the difference of the liquid level in the left side arm of the manometer between the beginning and the end of the run, and using the following equation the gas (carbon dioxide) produced during the reaction was calculated.

$X = h_x K$

where; X = μl. of gas produced at 0°C, 760 mm Hg pressure.

h = Change in reading of left side arm of the manometer.

K = Flask constant.

In order to get a precise determination, a blank test with distilled water was run.

Somogyi Micro Copper Method for glucose determination

The Somogyi method was used for the quantitative determination of carbohydrate.

Reagents:

Solution A (Alkaline Copper reagent): Rochelle salt, $KNaC_4H_4O_6 \cdot 4H_2O$, (90 g), and Tribasic sodium phospate dodecahydrate, $Na_3PO_4 \cdot 12H_2O$, (225 g) was dissolved in 700 ml of hot water. Cupric sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$, (30 g) was dissolved in about 100 ml of water. Potassium iodate, KIO_3 (3.5 g) was dissolved in a little water. The solutions were combined and made up to a total volume of 1000 ml.

Solution B: 90 g of Potassium oxalate, $K_2C_2O_4 \cdot H_2O$, and 4 g of Potassium iodide, KI, was dissolved in water and made up to a total volume of one liter.

Starch solution (Indicator): A 1% soluble starch solution was

prepared.

Procedure:

1) 10 ml of solution A was added to 20 ml of a sample (containing 5-15 mg of glucose) in a 300 ml Erlenmeyer flask.

2) The mouth of the flask was closed with a glass marble, and the flask was heated on a hot plate (making certain that the heat was great enough to boil the solution within two minutes). The solution was boiled for exactly three minutes, the flask was then cooled in a water bath. Care was exercised so that the flask was not shaken during the cooling period.

3) Ten ml of both solution B and 2 N H_2SO_4 solution were added and the flask was gently shaken until the red precipitate dissolved.

4) The solution was titrated with 0.05 N $Na_2S_2O_3$ solution. When the color of the solution turned to green, 4 drops of starch indicator were added. The titration was continued until a light blue color appeared. The volume of the $Na_2S_2O_3$ solution used was read from the burette.

5) A distilled water blank was also run.

In order to obtain the concentration of glucose in the sample a standard curve of milligrams of glucose vs. actual titrant (blank minus the titratant) was prepared. For the more precise work, the standard curve had to be calibrated in each seven to ten days.

IV. RESULTS

In the 500 ml shake flask fermentation experiments, six flasks were run. The medium in each flask was the same, except for the biotin concentration. The concentration of biotin in No. 1 and 2 had 1.25 γ/l; No. 3 and 4 had 2.5 γ/l; No. 5 and 6 had 10.0 γ/l.

Micrococcus glutamicus T. was inoculated into the shake flask medium. Twenty five hours after inoculation, 4 ml of 25% urea solution was added to flasks No. 2, 4, and 6.

The concentration of glucose and glutamic acid and the pH of the media were determined as shown in Table 10 to 15 and also plotted in Figure 11 to 17.

A qualitative analysis of the fermentative broth by paper chromatography is shown in Figure 17. Besides glutamic acid and its amide, glutamine, there was another amino acid. The spot was located above glutamic acid. Since the R_f value of glutamic acid under our conditions did not correspond with the literature value, the R_f of the unknown had to be corrected. The counted R_f value agreed most closely to that for leucine.

Table 10. Results for Flask No. 1. (Biotin Conc. 1.25 γ/l. No urea added during the fermentation.)

Time hr.	pH	Glucose g/dl	Glutamic Acid mg/ml
0	7.52	11.22	0.00
6	7.82	9.57	-
11	8.11	8.68	-
19	7.21	7.48	-
25	7.08	6.68	0.39
30	5.62	6.67	0.52
35	5.28	6.65	0.94
43	5.18	6.60	1.98
49	5.12	6.60	2.36

Table 11. Results for Flask No. 2. (Biotin conc. 1.25 γ/l. Urea added during the fermentation.)

Time hr.	pH	Glucose g/dl	Glutamic Acid mg/ml
0	7.52	11.22	0.00
6	7.97	8.51	-
11	8.18	8.19	-
19	7.32	7.50	-
25	7.00	7.09	0.35
		4 ml of 25% urea added	
30	8.46	7.09	0.49
35	8.42	4.54	1.76
43	8.21	3.52	2.81
49	8.22	2.94	3.05

Table 12. Results for Flask No. 3. (Biotin conc. 2.5 γ/1. No urea added during the fermentation.)

Time hr.	pH	Glucose g/dl	Glutamic Acid mg/ml
0	7.52	11.22	0.00
6	7.97	8.41	-
11	8.19	-	-
19	6.80	7.19	-
25	5.72	6.76	0.74
30	5.41	6.65	0.91
35	5.39	6.46	1.40
43	5.49	6.38	2.78
49	5.42	6.16	3.27

Table 13. Results for Flask No. 4. (Biotin conc. 2.5 γ/1. Urea added during the fermentation.)

Time hr.	pH	Glucose g/dl	Glutamic Acid mg/ml
0	7.52	11.22	0.00
6	7.81	8.69	-
11	8.20	-	-
19	6.71	7.02	-
25	5.70	6.79	0.76
	4 ml of 25% urea solution added		
30	8.19	4.21	0.71
35	7.99	2.61	2.94
43	7.19	1.39	4.45
49	6.52	0.66	5.07

Table 14. Results for Flask No. 5. (Biotin conc. 10.0 γ/l. No urea added during the fermentation).

Time hr.	pH	Glucose g/dl	Glutamic Acid mg/ml
0	7.52	11.22	0.00
6	8.00	8.62	-
11	8.12	8.38	-
19	6.72	6.93	-
25	5.65	6.78	0.68
30	5.80	6.49	0.87
35	5.31	6.30	1.14
43	5.20	-	1.40
49	5.20	6.23	1.65

Table 15. Result for Flask No. 6. (Biotin conc. 10.0 γ/l. Urea added during the fermentation).

Time hr.	pH	Glucose g/dl	Glutamic Acid mg/ml
0	7.52	11.22	0.00
6	7.94	8.61	-
11	8.12	8.39	-
19	6.61	7.86	-
25	5.81	6.49	0.25
	4 ml of 25% urea solution added		
30	8.31	3.69	0.69
35	7.96	2.98	1.27
43	7.39	1.10	1.77
49	7.03	0.56	1.93

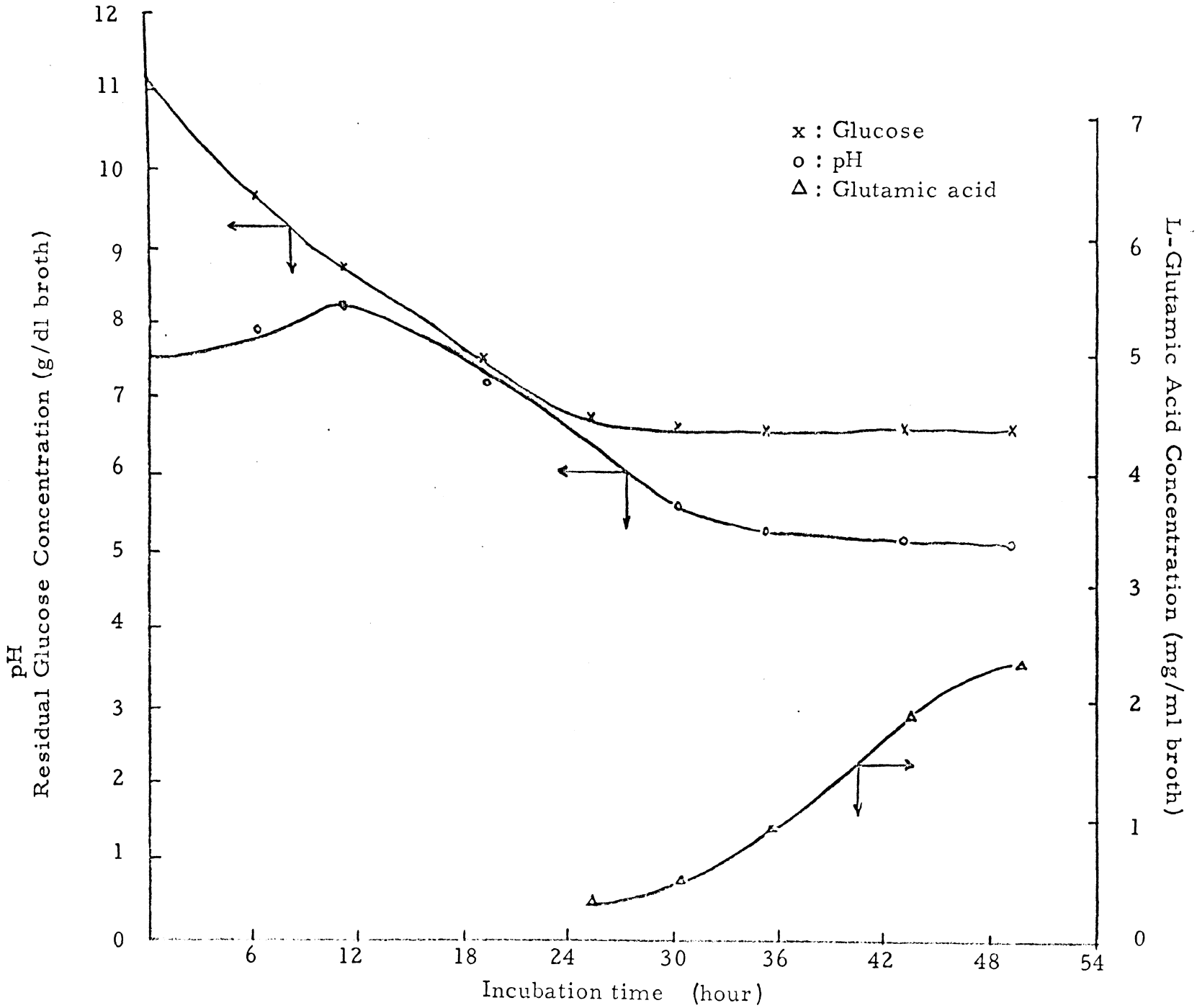
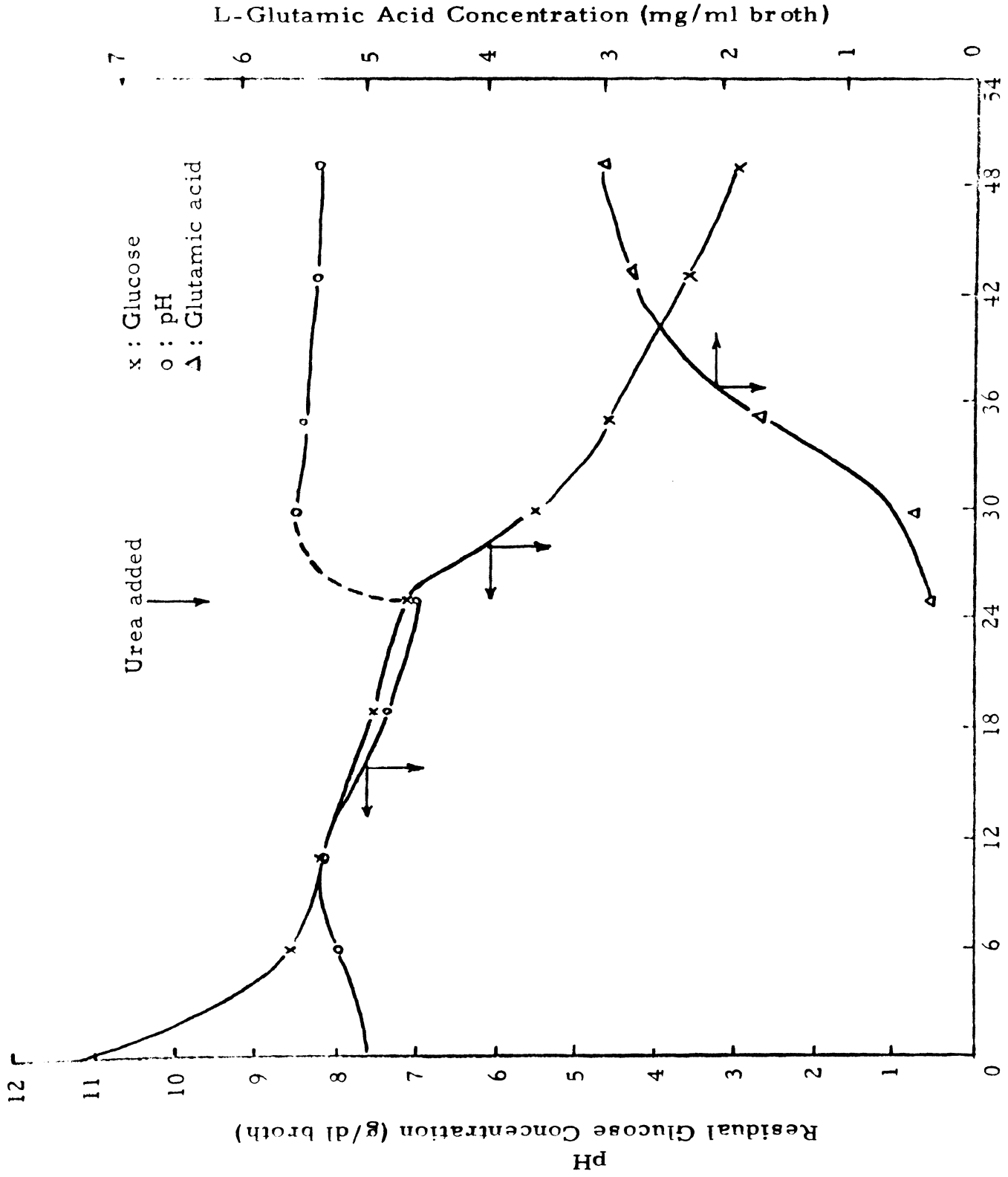


Figure 12. Results for Flask No. 1 Biotin conc. 1.25 γ/l


L-Glutamic Acid Concentration (mg/ml broth)
x : Glucose
o : pH
Δ : Glutamic acid
Urea added
pH
Residual Glucose Concentration (g/dl broth)

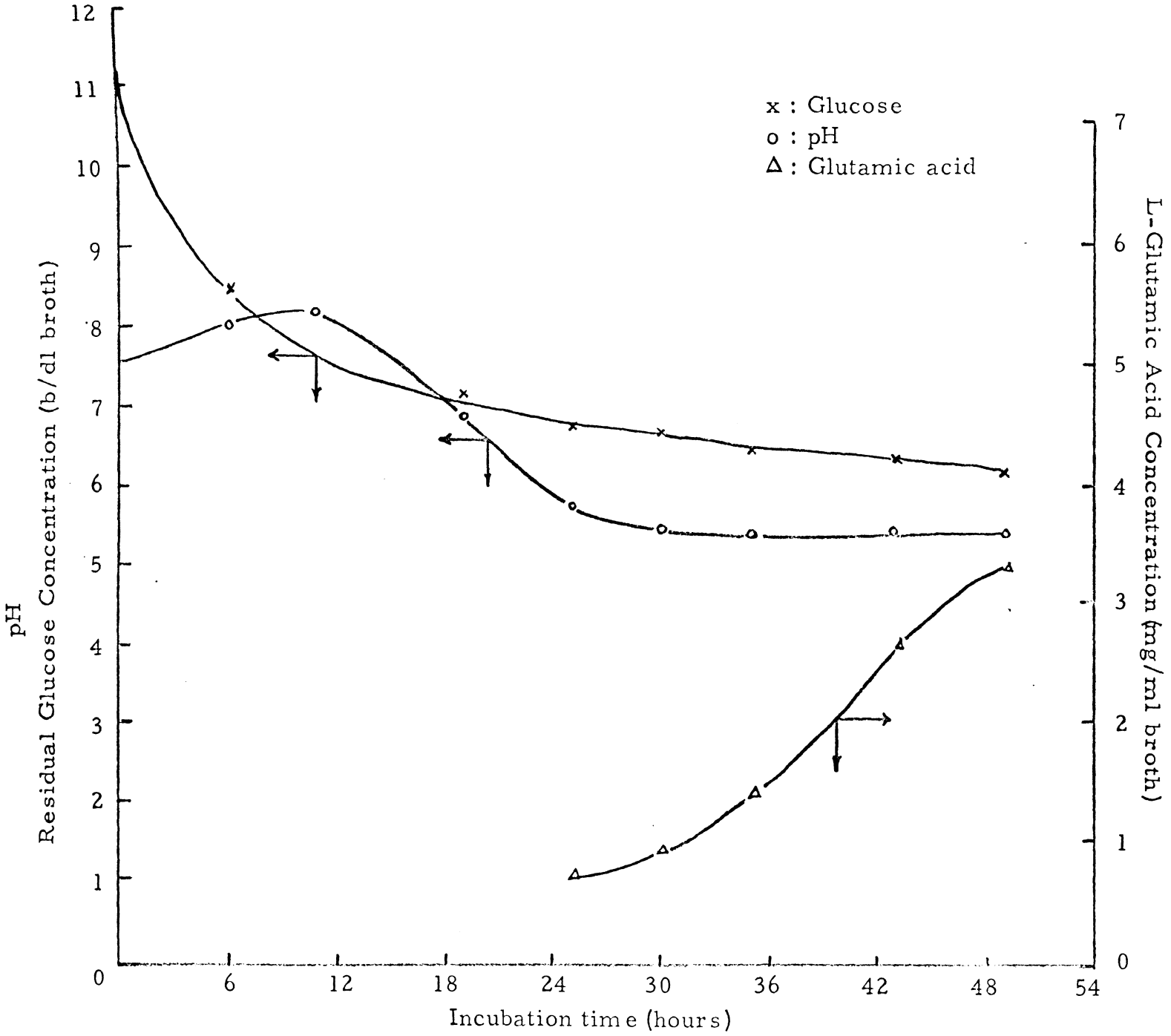


Figure 14. Results for Flask No. 3 Biotin conc. 2.5 γ/l

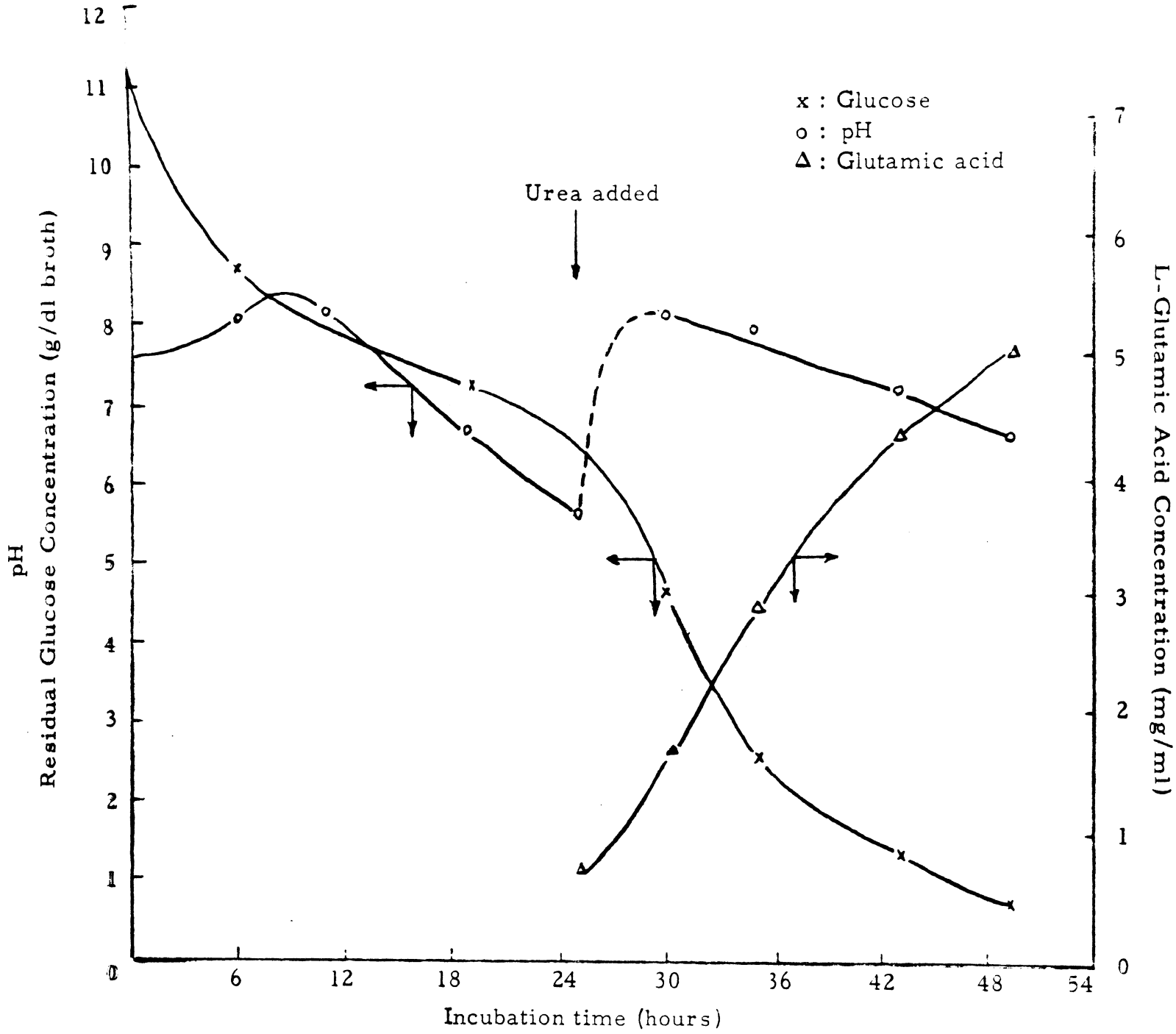


Figure 15. Results for Flask No. 4 Biotin conc. 2.5 γ/l, urea feeding

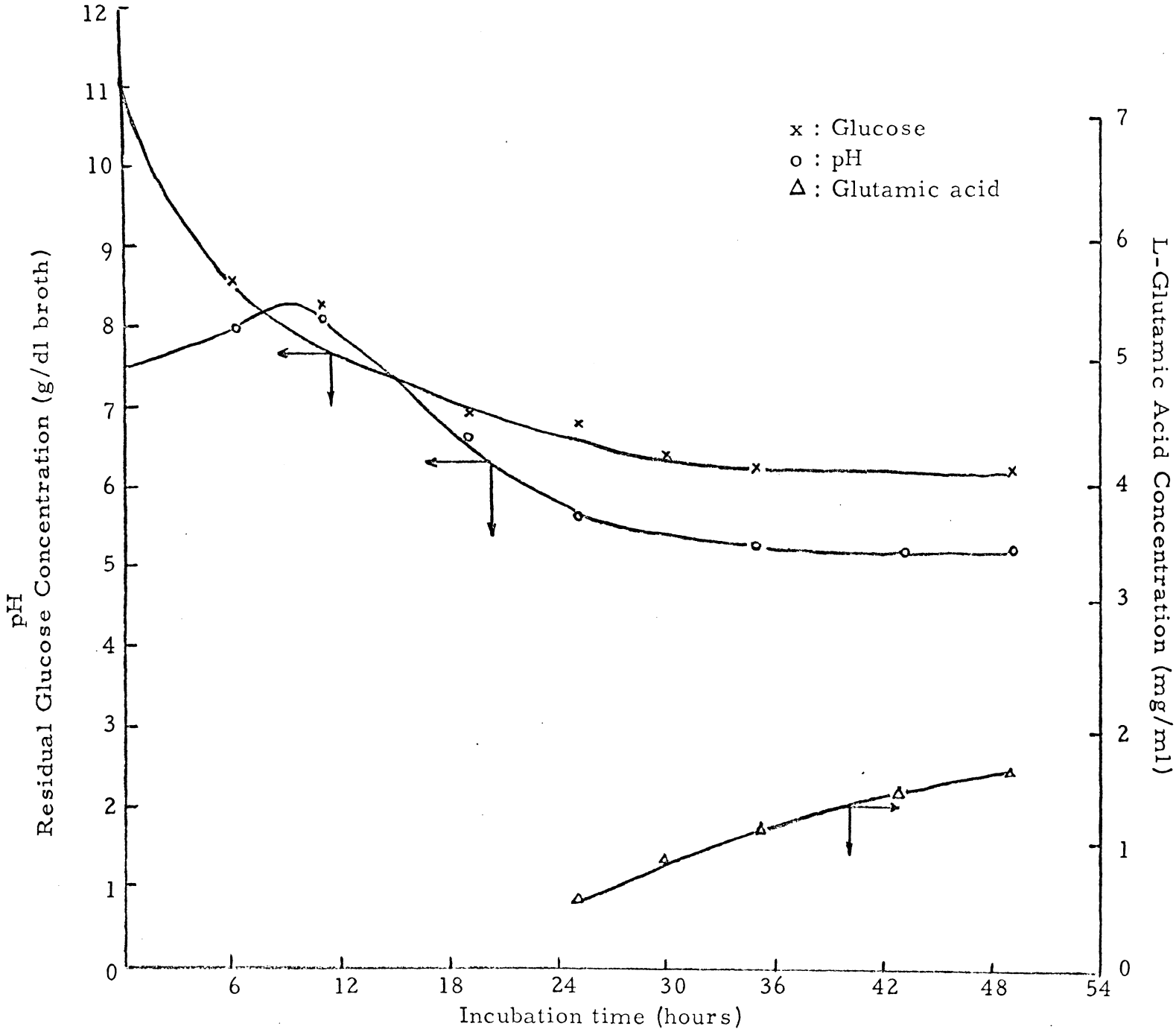


Figure 16. Results for Flask No. 5 Biotin conc. 10.0 γ/l

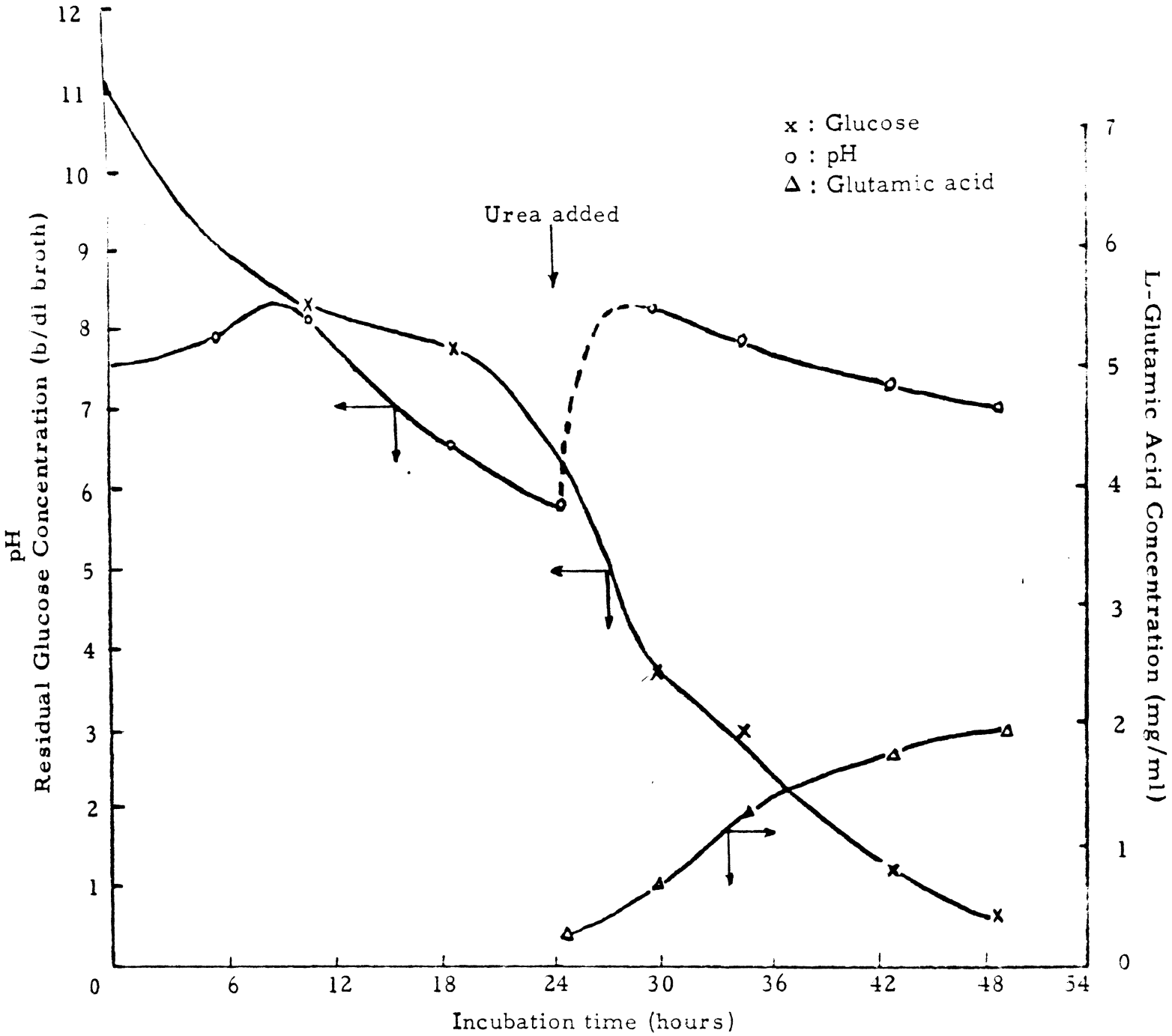


Figure 17. Results for Flask No. 6 Biotin conc. 10.0 γ/l, urea feeding

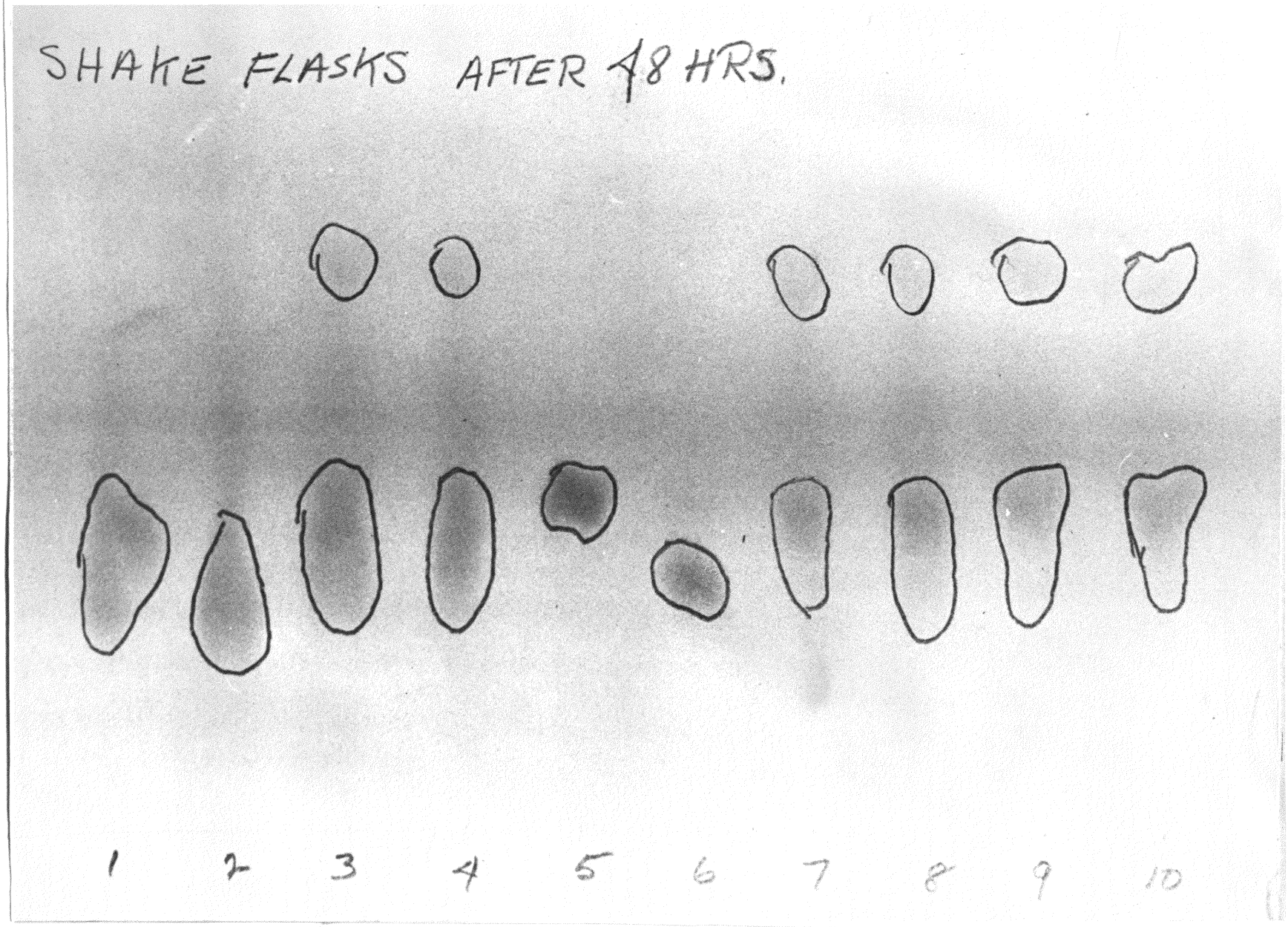


Figure 18. A typical Example of a Paper Chromatograph of the Amino Acids in a Fermentation Broth. (No. 5, Glutamic Acid, No. 6 Glutamine, known)

Three different fermentation experiments were conducted using the 5-1 Fermentor. The medium in each run was the same (see Table 9), except for the biotin concentration. In Run 1 the biotin concentration was 1.25 γ/1; in Run 2. it was 10.0 γ/1; and in Run 3. it was 2.5 γ/1. The impeller speed for Run 1 and 2 was 670 rpm and 420 rpm for Run 3. The aeration rate for the three runs was kept at 4 CFH .

The pH, temperature and aeration were recorded and the residual glucose, glutamic acid concentration were determined. The results were tabulated in the Tables 19, 20, and 21 and were plotted in Figures 18, 19, and 20.

The dissolved oxygen concentration and the oxygen uptake tests were performed during the fermentations. The dissolved oxygen was expressed in terms of percentage saturation of oxygen in the broth vs. incubation time (hours). The results of the experiments are shown in Figures 21, 22, 23.

Because of the difficulty experienced in determination of the cell count, the oxygen uptake rates were calculated in terms of millimoles of oxygen per hour for the total number of micro-organisms in the fermentor. The calculated values were as follows. An actual example of the calculation was shown in Appendix.

Table 16. The Oxygen Uptake Test for Run 1

Temperature: 30°C
Biotin Concentration: 1.25 γ/l
Aeration: 4 CFH
Agitation: 670 rpm
Time for dissolved oxygen to decrease from saturated to zero concentration: 14 hours.

Incubation Time (hr.)	R'_{O_2} *
1	1.73
4	1.45
7	2.19
10	33.10
12.5	42.21

* Rate of dissolved oxygen consumption, milli-moles of oxygen taken up by the volume of medium containing micro-organism in the fermentor per hour.

Table 17. The Oxygen Uptake Test for Run 2

Temperature: 30°C
Biotin concentration: 10.0 γ/l
Aeration: 4 CFH
Agitation: 670 rpm
Time for dissolved oxygen to decrease from saturated to zero concentration: 10.5 hours.
Oxygen uptake test had not been done on this run.

Table 18. The Oxygen Uptake Test for Run 3

Temperature: 30°C
Biotin concentration: 2.5 γ/l
Aeration: 4 CFH
Agitation: 420 rpm
Time for dissolved oxygen to decrease from saturated to zero concentration: 7.25 hours.

Incubation Time (hr.)	R'_{O_2}*
0.5	0.86
3.5	7.59
5.6	15.55
6.16	21.10

* Rate of dissolved oxygen consumption, milli-moles of oxygen taken up by the volume of medium containing micro-organism in the fermentor per hour.

Table 19. Results for 5-l Fermentor Fermentation Run 1

Biotin Concentration: 1.25 γ/l
Agitation: 620 rpm

Time hr.	pH	Temp. °C	Aeration CFH	Glucose g/dl	Glutamic Acid mg/ml
0	7.68	25.0	4	9.83	-
6	7.81	29.0	4	9.35	-
12	7.88	28.5	4	8.94	-
20	8.65	30.0	4	-	-
24	8.00	32.0	4	7.52	1.686
26	7.21	31.0	4	6.84	-
27	Urea added		4	-	-
28	8.20	-	4	-	-
30	8.75	-	4	6.26	3.970
34	8.61	31.0	4	-	-
36	8.71	30.0	4	5.94	5.16
39	8.72	29.0	4	5.56	-
48	8.65	27.0	4	5.32	6.89

Table 20. Results for 5-l Fermentor Fermentation Run 2

Biotin Concentration: 10.0 γ/l
Agitation: 620 rpm

Time hr.	pH	Temp. °C	Aeration CFH	Glucose g/dl	Glutamic Acid mg/ml
0	7.73	28.0	4	9.26	-
6	8.10	30.5	4	9.03	-
12	7.50	32.2	4	7.64	-
13	7.30	-	4	-	-
13.5	Urea added		pH adjusted to 7.88		-
15	Urea added		pH adjusted from 7.55 to 8.00		-
18	8.50	32.0	4	3.90	1.13
20	8.07	32.2	4	-	-
21	-	-	4	3.31	1.33
26	7.60	32.0	4	1.03	-
27	Urea added		pH adjusted to 8.00		-
31	8.58	31.5	4	0.20	3.74

Table 21. Results for 5-l Fermentor Fermentation Run 3

Biotin Concentration: 2.5γ/l
Agitation: 420 rpm

Time hr.	pH	Temp. °C	Aeration CFH	Glucose g/dl	Glutamic Acid mg/ml
0	7.88	29.5	4	9.75	-
3.5	7.93	30.0	4	9.68	-
6	8.00	30.0	4	9.63	-
10	8.27	31.0	4	8.80	-
12	8.17	30.0	4	8.49	-
15	7.39	29.8	4	8.00	-
15.5	Urea added		pH adjusted from 7.30 to 7.50		-
19	8.10	-	4	-	-
20	Urea added		pH adjusted from 6.70 to 7.50		-
21	8.10	30.0	4	6.19	0.645
26	8.35	27.0	4	4.95	-
31	8.21	31.0	4	3.58	2.79
36	7.98	30.0	4	0.80	-
45	5.87	31.0	4	0.72	4.45

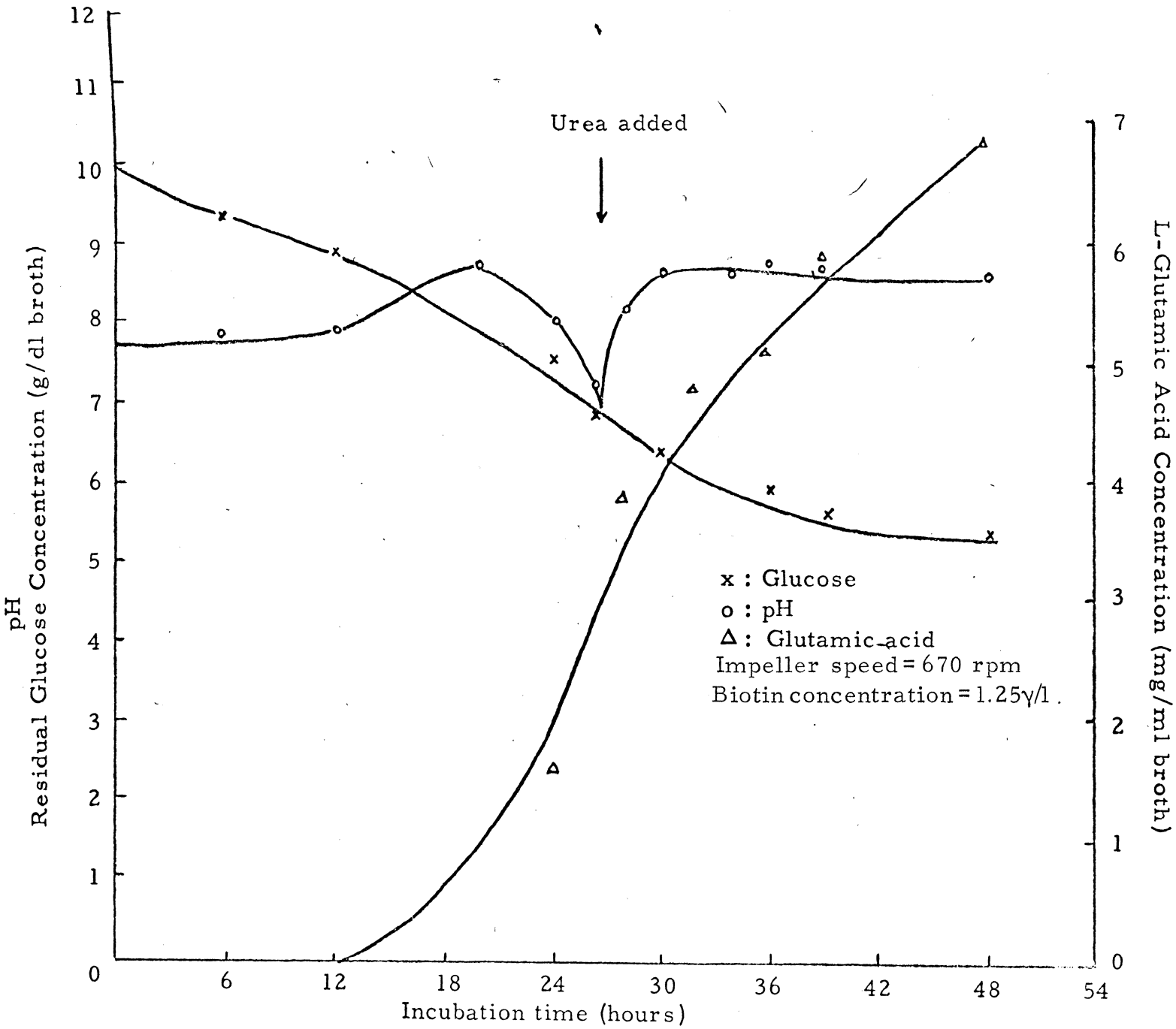


Figure 19. Results for Run 1. in 5-1 Fermentor

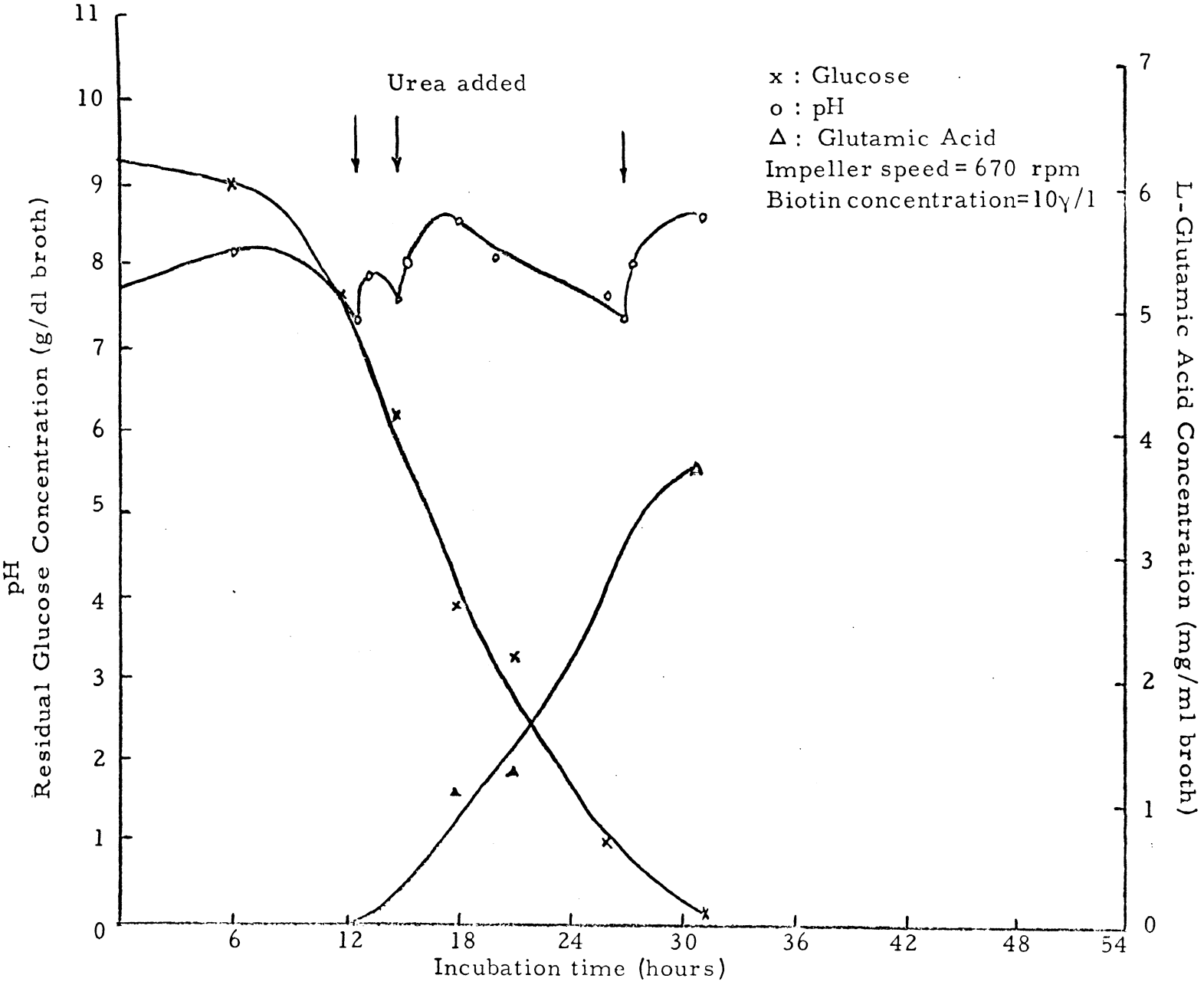


Figure 20. Results for Run 2. in 5-1 Fermentor

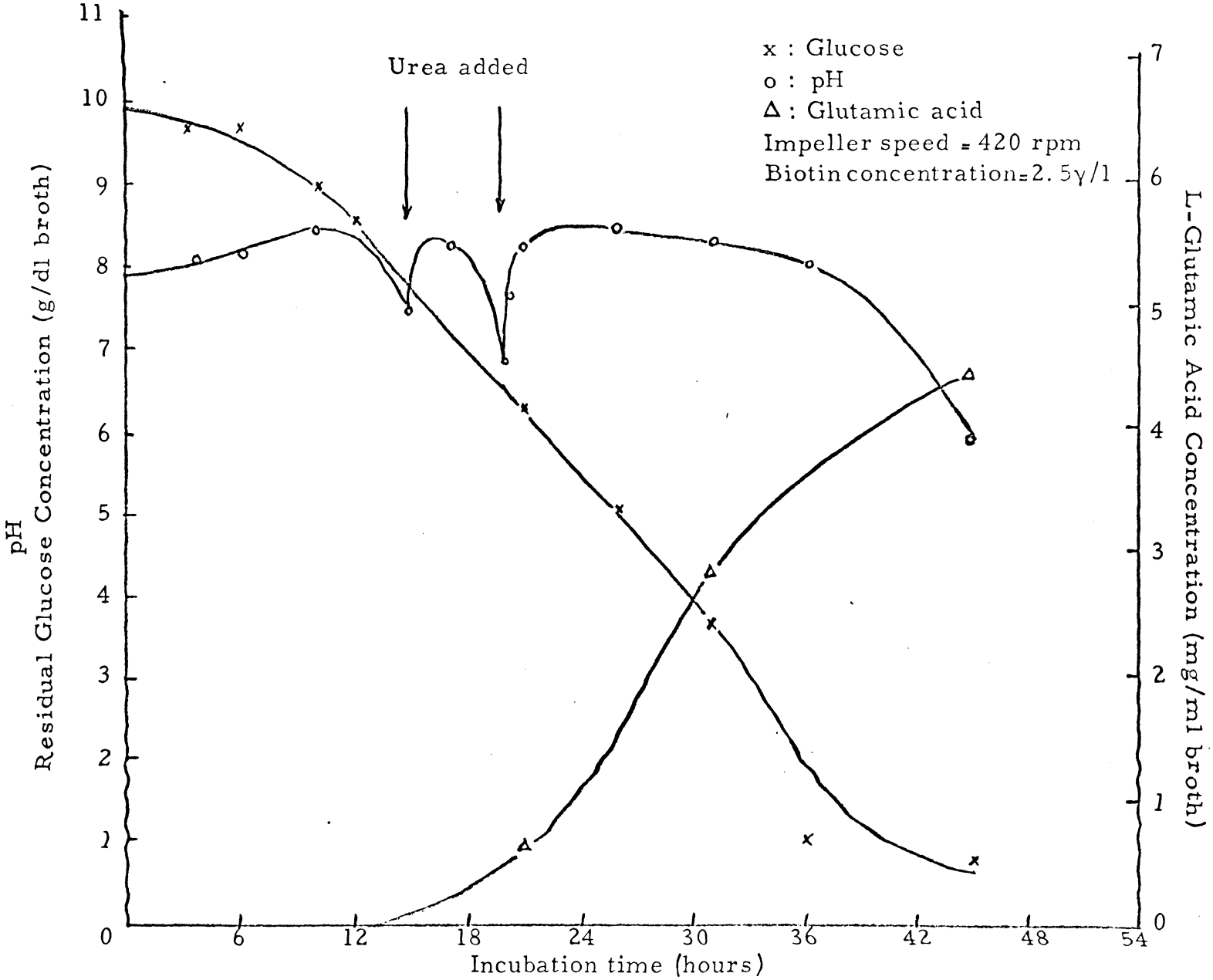


Figure 21. Results for Run 3. in 5-l Fermentor

Impeller speed = 670 rpm
Biotin concentration = 1.25 γ/l

Test	Time hr.	R'_{O_2}
1	1	1.73
2	4	1.45
3	7	2.19
4	10	33.10
5	12.5	42.21

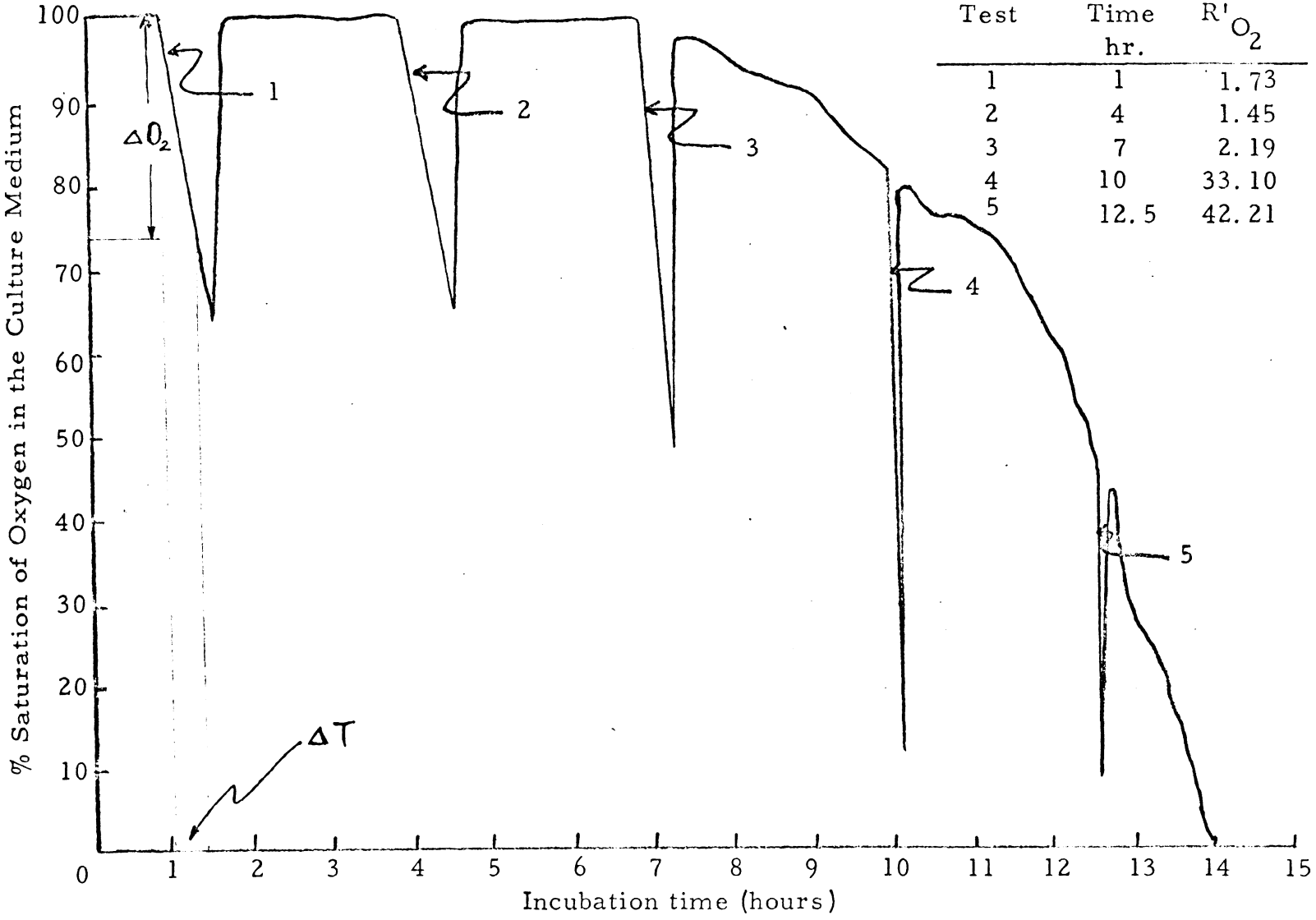


Figure 22. Dissolved Oxygen Concentration in the Culture Medium of 5-l Fermentor for Run 1.

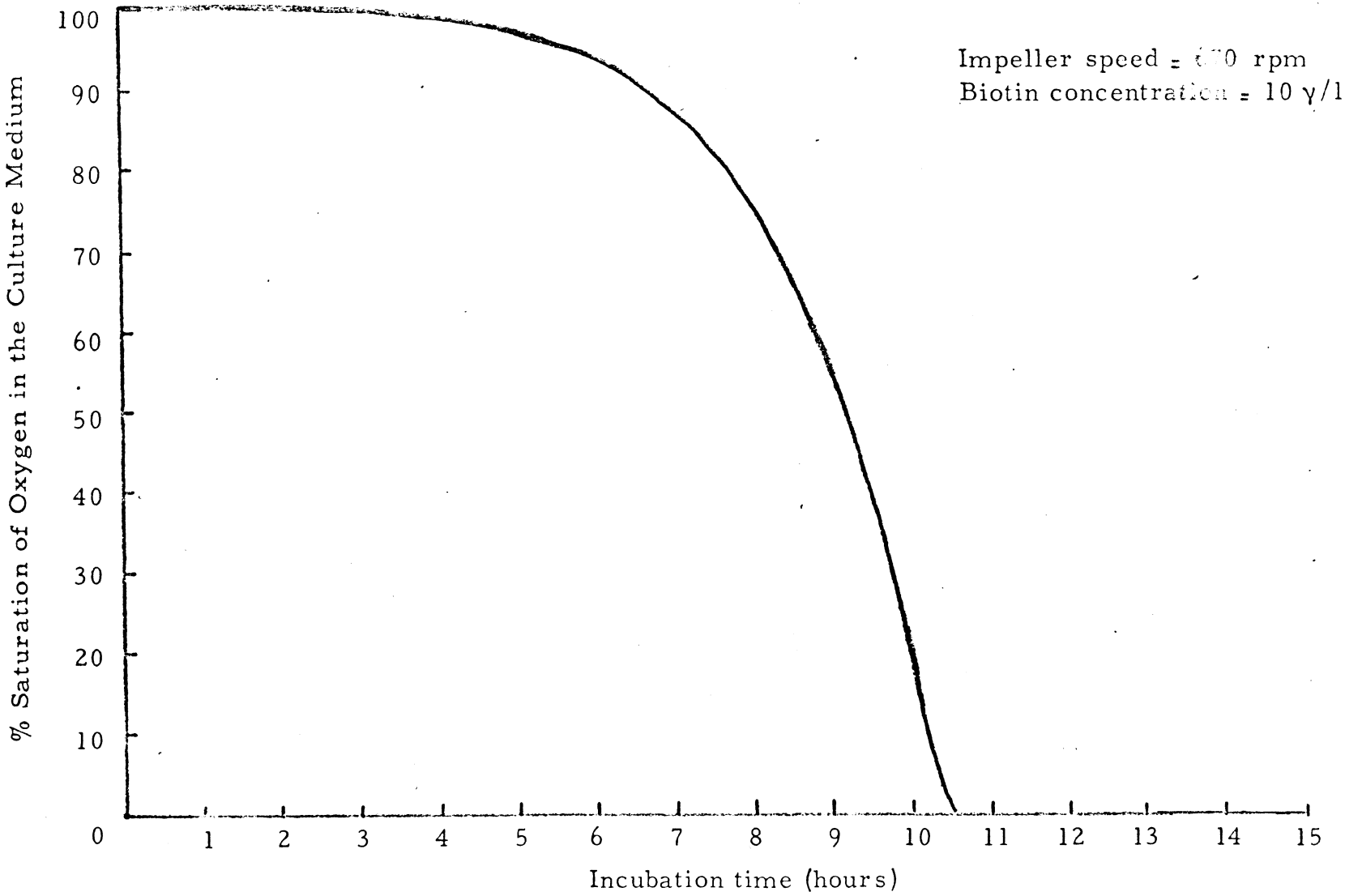


Figure 23. Dissolved Oxygen Concentration in the Culture Medium of 5-1 Fermentor for Run 2

Impeller speed = 420 rpm
Biotin concentration = 2.5 γ/l

Test	Time hr.	R'_{O_2}
1	0.5	0.86
2	3.5	7.59
3	5.6	15.55
4	6.16	21.10

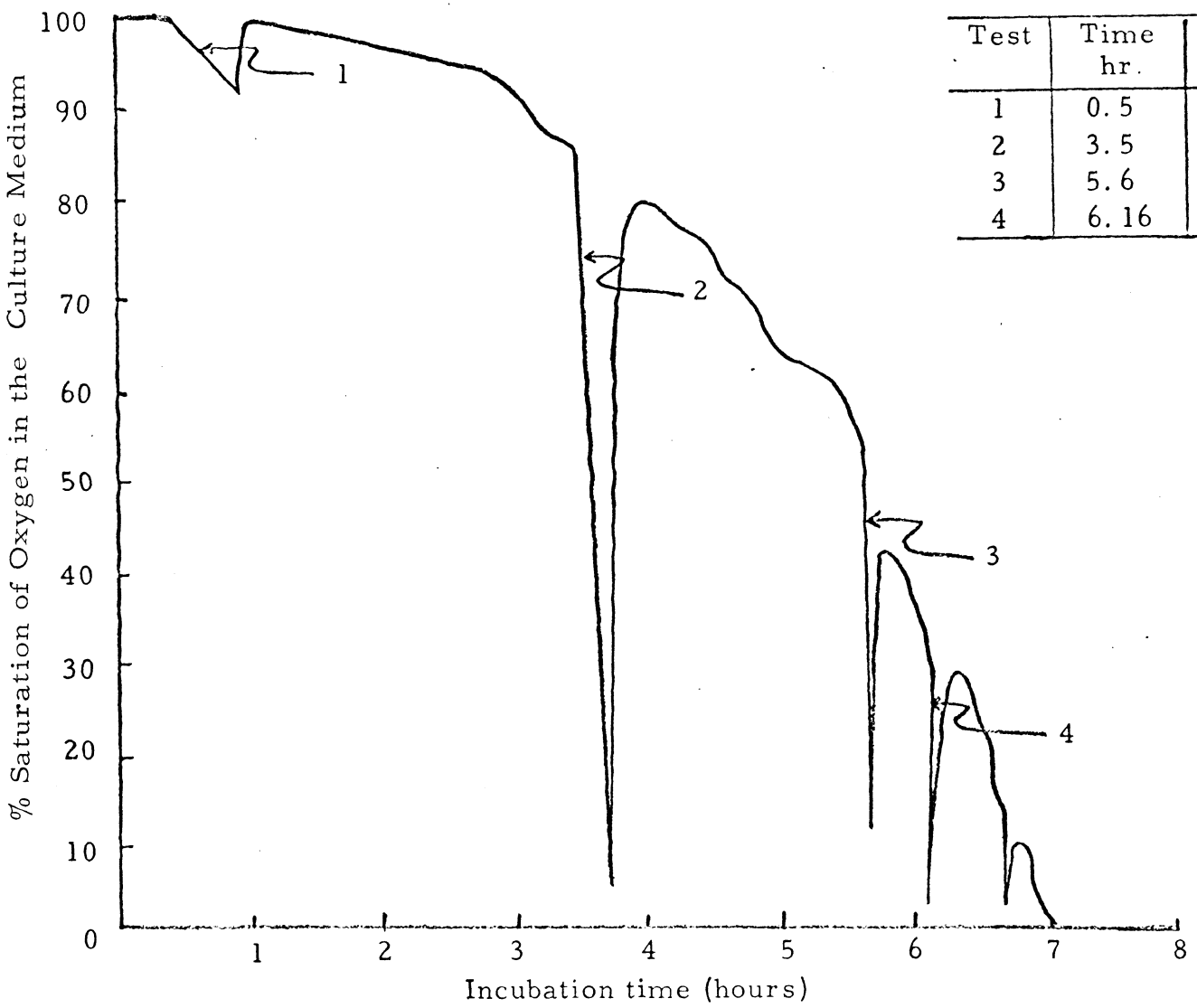


Figure 24. Dissolved Oxygen Concentration in the Culture Medium of 5-l Fermentor for Run 3

V. DISCUSSION

Micrococcus glutamicus obtained from Northern Regional Research Laboratory was tried as a glutamic acid producer. Though different media and conditions were tried in shake flask experiments, only a low concentration of glutamic acid, 0.35 to 0.40 mg of glutamic acid per milliliter of fermentative broth, was produced. It might be that the strain itself is not a good glutamic acid producer, or that optimum conditions for producing glutamic acid by this micro-organism were not used. Instead of using this strain, another strain obtained from Wei Chaun Foods Co. (not a commercial strain) was tried. The results described in this thesis were obtained for the most part with this second strain.

In both shake flasks and in the five liter fermentor, the pH of the media usually rose during the first twelve hours, and then went down. Also, whenever urea was added to the media the pH rose slowly and reached its highest value in one or two hours after the addition and then went down again. This is because when the urea in the medium is utilized by the micro-organism it is first decomposed to ammonium ion, and, the pH of the medium increases. When the ammonium ion was utilized in glutamic acid formation, the acidity of the amino acid caused the pH to decrease.

In both the shake flasks and the five liter fermentor, it was

shown that the glucose consumption increased when the biotin concentration was increased. This is because biotin is a growth factor which controls the formation of the micro-organism. In a biotin rich medium most of the glucose was used as a nutrient and energy source for cell propagation. But, in a low biotin medium a small part of glucose was consumed as an energy source and the rest as a raw material for glutamic acid formation. Therefore, in order to get a high yield of glutamic acid a suboptimal concentration of biotin must be used. From the glutamic acid data in shake flasks, it seems an optimum glutamic acid production is obtained with a biotin concentration near 2.5 γ/l.

In the flasks where urea solution was not added during the fermentation the glucose consumption was very slow during the latter half of the fermentation. But in other cases where urea was added, the residual glucose decreased rapidly. This is because Micrococcus glutamicus prefers to grow in a slightly alkali medium. When pH of the medium decreased to 7 or lower the growth of the micro-organism was inhibited, and the rate of glucose consumption slowed down. On the other hand, though the rate of the consumption of glucose was stimulated by the addition of urea during the fermentation, the production of glutamic acid was not proportional to the glucose used. A portion of the remainder of the glucose was spent in the growth of the micro-organism and the remainder was for glutamic acid formation.

Glutamic acid usually started to accumulate twenty-four hours after the inoculation of the micro-organism. During the early period of the fermentation micro-organisms were formed which converted the glucose in the medium to glutamic acid in the latter half of the fermentation.

Glutamine, an amide of glutamic acid, was an important by-product of the fermentation. Glutamine was formed from glutamic acid. Therefore, the concentration of glutamic acid was low when it was determined by the manometric technique which was specified for glutamic acid. By paper chromatography it was shown that glutamine was produced only in the last part of the fermentation (after 36 hours). This occurred when a considerable excess of ammonium ions was present in the medium after the addition of urea. During this time the glutamic acid was converted to glutamine. Also on the paper chromatography there was another spot, located above the glutamic acid. By calculating the R_f value of this amino acid and comparing it with the R_f value of glutamic acid, obtained under the same conditions, the most possible amino acid for this spot is leucine.

If the fermentation broth was hydrolyzed with concentrated hydrochloric acid, the glutamine was converted to glutamic acid. Paper chromatography clearly showed that only one spot, with a R_f value equivalent to glutamic acid, appeared after the hydrolysis of the fermentation broth.

Foaming was a serious problem in this fermentation. Various kinds of antifoam were used. In one case the emulsion of the antifoam was destroyed when it was autoclaved (AF. 20 Silicone Antifoam). Some of the others did not control foaming effectively and an unusually large amount (75-100 ml) of the antifoam was required for a run (Sag 470 Silicone Antifoam Emulsion).

A mechanical foam breaker was tried. A comb like stainless blade was clamped to the agitator shaft one inch above the surface of the liquid medium. The blade controlled the foam during the early period of the fermentation when coarse bubbles were formed. But when the foaming increased during the accumulation of glutamic acid, the blade, instead of breaking the bubbles, agitated the medium more vigorously and caused an increase in foaming. If the rotating speed of the blade could be reduced by either using an extra shaft of low speed or some other means, this method might be a practical method of foam control.

Foaming started at the beginning of the fermentation, but during the first twelve hours the foam was not so thick. The diameter of each bubble was large and when the bubbles accumulated to a certain height over the liquid surface they broke by themselves and the foam disappeared for a while until new bubbles rose again. After twenty four hours, foam rose up vigorously and most of the antifoam

was added at this period. From the author's experience, twelve to twenty-four hours is the most dangerous period for the fermentation. If the antifoam is a poor one, or if there is insufficient antifoam in the reservoir or there is some mechanical failure in the foam control system, the broth in the fermentor will blow out and this will result in contamination and loss of media. After twenty-four hours, the foam was continuously produced from the medium, but it did not rise too far. It accumulated as a thick layer of small bubbles (about thirty second inch in diameter) on the surface of the medium. From shake flask experiments it was shown that foam was produced at a greater rate in biotin rich media than in biotin poor ones. The material which caused foaming is assumed to be protein of peptides in the media. It is clear that the amino acids start to accumulate during the first twelve to twenty-four hours, so that the foaming is serious in this period.

If one considers just Run 1 and 2 (both are at 670 rpm), it can be seen that in biotin rich media the dissolved oxygen decreases from saturated to zero faster than in biotin poor ones. This is because biotin is a growth factor which controls the generation of the micro-organisms in the fermentation. Therefore, in biotin rich media the micro-organisms grow faster and the larger number of organisms require more oxygen for their respiration.

In the oxygen uptake experiments, no useful cell count was

obtained because of some difficulties in experimental technique. So the oxygen uptake rate per cell count can not be obtained from those oxygen concentration charts. However the rate of the consumption of the dissolved oxygen by all the micro-organisms in the fermentor at a certain time can be calculated from the slope of the curve and expressed in terms of R'_{O_2}, milligram moles of oxygen per hour utilized per microbial population in the fermentor.

From the calculated values it shows that the R'_{O_2} increases with time and increases rapidly after six hours from the inoculation of the fermentation. This is due no doubt to the fact that the micro-organisms are not very active during the first six hours. The changing of the environment (from flask to fermentor) can cause a slowing down of the growth of the organisms. The micro-organisms recover their normal condition and start to grow rapidly after this initial period.

The oxygen analyzer used in the experiments is a very delicate instrument. But because of its construction the analyzer can not be autoclaved with the fermentor. For every run of the fermentation a risk of contaminating the medium must be taken in mounting the analyzer in the fermentor. If a special design is available which is resistant to high temperature and non-toxic to the micro-organism, the dissolved oxygen measurement can be done in a more convenient way and

contamination can be prevented.

According to Henry's law temperature controls the concentration of the dissolved oxygen (or other gases) in aqueous media. Therefore, in order to increase the precision in the oxygen uptake experiments and also to regulate the fermentation itself a temperature control device, which controls the temperature within ± 2°C, must be included in the fermentor assembly. The control system can be composed of a solinoid value attached to the inlet line of the cooling finger, a heating element either put into the inside of the fermentor or wound on the glass vessel and a termperature sensor and controller.

VI. CONCLUSION

1. Micrococcus glutamicus obtained from NRRL produced only a very low concentration of glutamic acid, 0.35-0.4 mg/ml.

2. Micrococcus glutamicus T. produced a maximum concentration of glutamic acid of 5.07 mg/ml in shake flask and 6.89 mg/ml in a 5-1 fermentor.

3. Biotin affects the production of glutamic acid. The optium biotin concentration for glutamic acid formation in a shake flask is near 2.5 γ/1.

4. Glucose in the medium is both an energy source and a raw material for glutamic acid synthesis. Therefore, a decrease in glucose is not proportional to the increase in glutamic acid production.

5. When the pH is controlled by urea the formation of glutamic acid will increase and the residual glucose will decrease to a very low concentration.

6. Foaming is serious when amino acids start to appear in the medium; twelve to twenty-four hours from the time of inoculation of the micro-organism.

7. The rate of dissolved oxygen consumption increases with the incubation time.

8. It requires about six hours for the micro-organism to adapt after being transferred from a shake flask to a fermentor.

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VIII. APPENDIX

Calculation of the Rate of Dissolved Oxygen Consumption R'_{O_2}

R'_{O_2} is determined by the following equation;

$$R'_{O_2} = S \times \frac{\Delta O_2}{\Delta T} \times V$$

Where;

R'_{O_2} = milli-moles of oxygen taken up by the volume of medium containing micro-organism in the fermentor per hour.

S = concentration of oxygen in 100% O_2 saturated water at the temperature and pressure of the fermentation (760 mm Hg 30°C). From the handbook (Deluca 1963) this value is 1.05×10^{-3} milli-mole/ml of H_2O

ΔO_2 = change in oxygen concentration during the uptake test expressed in percent saturation.

ΔT = period of aeration stopped in hours.

V = volume of the medium in milliliter in the fermentor containing the total number of micro-organism

Example: 5-l fermentor Run 1. after one hour. (see Figure 22.)

ΔO_2 = (100-72.5)/100 = 0.275

ΔT = 20 min = 1/3 hr.

V = 2000 ml

$R'_{O_2} = 1.05 \times 10^{-3} \times \frac{0.275}{1/3} \times 2000$

= 1.73 milli-mole of oxygen taken up by the volume of the medium containing micro-organism in the fermentor per hour.

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